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13. ABSTRACT *(Maximum 200 Words)*

We had earlier demonstrated the prophylactic and therapeutic efficacy of tumor derived heat shock protein, gp96-peptide complexes. Since this tumor rejection property was specifically mediated by *tumor derived* and **not** non-tumor derived gp96-peptide complexes, and that gp96 preparations stripped of its peptides are non-immunogenic, we examined the hypothesis whether prostate cancer associated peptides which may act as tumor rejection antigens can be identified in gp96-peptide complexes utilizing a combinatorial single chain phage display antibody library. We have successfully used combinatorial single chain phage display library (scFv) for the detection of tumor rejection antigens and to define the heterogeneity of cancer antigens in prostate cancer. These novel reagents helped us to characterization of the 170 kDa protein specifically expressed in MAT-LyLu cells that could be a target for immunotherapy. These scFvs were used to identify synthetic peptides that mimic the activity of the tumor rejection antigen gp96. We also demonstrated that a tumor protective immune response can be generated using these synthetic peptides. Our results now confirm that T cell defined epitopes can be recognized by antibodies and that there may exist considerable overlap. In this respect, the existing paradigm was challenged.

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Table of Contents

INTRODUCTION

The long term goal of our research is to develop active specific immunotherapy for prostate cancer using tumor associated antigens. Identification of tumor associated antigens in prostate cancer has been challenging. We proposed to overcome this challenge by identifying tumor associated peptides that may be tumor rejection antigens. The identification and isolation of prostate cancer associated antigens was undertaken using an innovative approach that utilized heat shock protein gp96 and the combinatorial antibody phage display library. Heat shock protein, gp96, was associated with peptides in the endoplasmic reticulum (ER) and the gp96-peptide complex had tumor rejection properties (1-4). Since the tumor rejection property was specific and could not be mediated by gp96 preparations from other tissues and that there was no sequence difference or mutation in gp96 genes from different tissues or species and that gp96 stripped of the peptides had lost tumor rejection properties, it was presumed that the imunogenicity resided in the peptide and /or gp96-peptide complexes. The role of gp96 was merely as a peptide chaperone with little or no relevance biological effect. In the examination of our hypothesis it served as a tool to isolate tumor rejection peptides. It is obvious that identification of these tumor associated peptides would be useful therapeutic and preventive agents. With this goal in mind, we examined the hypothesis if tumor specific immunogenic peptides can be identified, isolated and characterized using single chain phage display antibody library (scFv).

BODY

THE FOLLOWING IS THE STATEMENT OF WORK AND TIME SCHEDULE AS PROPOSED IN THE ORIGINAL APPLICATION AND THE WORK COMPLETED.

Task 1 : 0-12 months

Identification of prostate cancer associated antigen (PAA) in gp96-peptide complexes isolated from metastatic and non-metastatic prostate cancer cells.

This task has been *completed* as proposed. Differential panning of the combinatorial phage display single chain antibody library over metastatic (MAT-LyLu) and non-metastatic (Dunning G) and non -tumor liver tissue purified gp96-peptide complexes resulted in single chain antibodies (scFvs) that react to PAA specific to metastatic MAT-LyLu cells or non-metastatic Dunning G cells. Distinct set of antibodies react either to cell surface antigens or to intracellular antigens (purified gp96-peptide complexes). The construction of the phage display library is described in Figure 1 and the panning strategy used for the isolation of tumor specific gp96 reactive phages and soluble antibodies described in Figure 2 and for cell surface antigens in Figure 3.

A. Isolation of tumor specific single chain antibodies reactive to tumor derived gp96 and their reactivity.

(i) The single chain phage display library has a large repertoire of antibody diversity.

The synthetic phage display library which consist of single chain Fv (scFv) fragments of antibodies displayed on the surface of filamentous bacteriophages was a gift from Dr. G. Winters, MRC Center for Protein Engineering, Cambridge, UK) and is described by Nissim

et. al. (5). This library utilizes 50 human germline V_H segments assembled *in vitro* with random synthetic 4-12 residue long CDR3. The V_H segments were cloned into phagemid vector pHEN1 carrying a human Vλ3 light chain to generate a repertoire of $>10^8$ clones (Figure 1). The scFv fragments can be expressed on phages and in soluble form. In both libraries the scFv fragments have a c-myc tag facilitating the detection of phages carrying the desired antibody, utilizing the anti-c-myc mAb9E10.

(ii) Panning strategy for the identification of tumor derived gp96-peptide complexes.
Gp96-peptide complexes purified from MAT-LyLu and liver were the source of the protein that was used to identify phages that specifically reacted to tumor derived gp96-peptide. Several different concentrations of the purified complexes was tested ranging from 10 to 100μg per mL. The panning strategy (Figure 2) was designed such that non-specific phages reacted to liver derived gp96-peptide could be eliminated. Amplification of the phages was undertaken only after sufficient depletion of the phage library with respect to non-tumor derived phages had already taken. The unabsorbed phages were allowed to bind to tumor derived gp96-peptide complexes, eluted, amplified and then tested. For the detection of cell surface antigens the strategy was not of depletion but of affinity selection and bound phages were eluted (Figure 3) which were amplified and passed over Dunning G for reactivity.

(iii) Isolation of tumor specicfic scFvs
The reactivity of the eluted phages was screened with MAT-LyLu and liver derived gp96-peptide complexes using the enzyme linked immunosorbent assay (ELISA) and this can be easily done using the 9E10 antimyc antibody as the second antibody and the absorbance read at 490/403 nm. Our results (Figures 4-6) clearly shows that phages of differential reactivity exists in the library and can be separated out. We **successfully** identified phages that react specifically to MAT-LyLu derived gp96-peptide complexes (Figure 7), however, phages that react with both liver and tumor derived gp96-peptide complexes (Figure 5) or phages that react only with liver (Figure 6) were also identified. These experiments were consistent in at least more than three different purified preparations of gp96-peptide complexes. None of these phages reacted with gp96 native protein itself. These results clearly indicate that tumor specific scFvs exist in the library and can be identified from tumor and tissue derived gp96-peptide complexes. To further test the specificity of these scFvs and to characterize the cell surface antigens from metastatic cell line, MAT-LyLu we isolated cell surface antigens reactive phages. We also used gp96-reactive phages to react with intact cells and the results are shown in Figures 8-10. Gp96 reactive scFvs did not react with intact prostate cancer cells (Figure 8) ; cell surface reactive phages panned over MAT-LyLu showed differential reactivity with MAT-LyLu and Dunning G (Figure 9) [Note that MAT-LyLu is a metastatic variant of Dunning G cell line]; MAT-Ly Lu panned cell surface scFvs did not react to gp96-peptide complexes (Figure 9); Cell surface scFvs panned over MAT-LyLu showed little reactivity to human prostate cancer cells, TSU (Figure 10)

Conclusion : Tumor specific scFvs reactive to either gp96-peptide complexes or to cell surface can be isolated and their reactivity is specific. Our panning strategy gave adequate number of scFvs for a comprehensive analysis.

(iv) **Sequence analysis of the scFvs**
Several of these scFvs have been sequenced and V_H -CDR3 sequence of four of them are presented in Table 1 . Two of the scFvs (E6 and F3 have the same CDR3 sequence and are of DP-32 germline which is different from the germline (DP-38) of cell surface specific scFvs (Table 1).

TASK 1 IS COMPLETE AS PROPOSED IN THE STATEMENT OF WORK

- specific gp96 protein is associated with peptide and the peptides associated is reflection of the antigenic repertoire of the tissue from which the gp96 preparations are derived. The phage antibodies may be directed against the peptides or recognize a specific conformation of gp96 and a distinct peptide

- the synthetic combinatorial phage display antibody library can be used to differenially separate out the array of antigenic repertoire of a specified tissue.

- several rounds of panning over non-tumor derived gp96-peptide complexes is still not sufficient to completely deplete phages that react specifically only to non-tumor derived gp96-peptide complexes or that react with equivalent avidity to tumor and non-tumor derived gp96-peptide complexes.

Task 2: 10-24 months

Identification of the proteins of origin of the peptides isolated from prostate cancer cells.

This task is *complete* using two of the scFvs and one protein identified specifically in MAT-LyLu cells

(i) **Identification of cell specific protein using scFvs**
Identification of a 170 kDa protein specifically expressed in MAT-LyLu cells is presented in Figures 11 and 12. These scFvs panned over the tumor cell line MAT-LyLu and specifically reactive to MAT-LyLu derived gp96-peptide complexes can immuno-precipitate cell specific protein as determined by [^{35}-S] methionine metabolic cell labeling [Figures 11 and 12]. A 170 kDa protein is detected in MAT-LyLu cells and **not** in Dunning G [MAT-LyLu cells are a derivative of Dunning G cells]. This was confirmed by immuno-precipitation (IP) of cell lysates by soluble scFv E6, both by metabolic labeling [Figure11] and by staining of electrophoresed IPs by a sensitive Zinc based reagent [Figure 12, Pierce, Rockford, IL]. Both E6 and F3, panned over MAT-LyLu cells, though from distinct phage clones showed similar reactivity and their CDR3 regions are identical which when sequenced corresponded to GKYIRSV of germ line DP-32 origin (Table 1).

(ii) **Structural analysis of the cell specific 170 kDa protein**
The isolated immune complexes are subjected to an acid digestion and the gp96 and peptides

separated by Amicon filtration as described in (49,50) and the matric assisted laser desorption ionization mass spectrophotometry (MALDI-MS) peptide mass mapping analysis performed (Figures 13-15). The sequence of the peptides is searched using NCBI and Genpept data bases and match profile suggested that this protein in myosin, heavy polypeptide 9, non-muscle.

(iii) Isolation of synthetic peptide mimotopes of gp96-peptide complexes using tumor specific scFvs

Since several of these scFvs may react to conformational epitope(s) of tumor derived gp96-peptide complexes it was necessary to examine if these scFvs would react to synthetic peptides in a combinatorial phage display peptide library. These peptide antigens would be conformational mimics of *in vivo* gp96-peptide complexes and thus it would be possible to synthesize standardized immunogens. Two such peptide libraries, LX-8 (consists of conformational epitopes of 12 amino-acids, cysteine each at positions 2 and 11) and X-15 (15 amino-acid linear epitope) were reacted to scFv E6 (tumor specific) and B11(non reactive to tumor gp96-peptide complex). Panning strategy is outlined in Figure 16. The scFv E6, that showed specific reactivity to tumor derived gp96-peptide complexes also reacted to synthetic peptides from *a combinatorial phage display* ***peptide*** *library (6,7). These peptides are presumably mimics of tumor derived gp96-peptide complexes.* Reactivity of scFv E6 to a set of peptide phage clones from the two libraries after four rounds of enrichment is shown in Figures 17 and 18. B11 is an MAT-LyLu derived non-reactive to gp96-peptide phage control. The reactivity of a set of peptide phage clones to the two libraries shown in Figures 19 and 20 and the sequence of some of the synthetic peptides that showed highest reactivity is shown in Table 2. These peptides are synthetic mimotopes to tumor specific gp96-peptide complexes. Several of these phage clones showed similar peptide sequence indicating a selection and enrichment of immuno-dominant peptide epitopes. One such representative peptide from LX-8 having the sequence, YCQEGDSPRLCL (BTE6-LX8b), and one from X-15, GQWQSGDRYWMETST (BTE6-X-15-7) were further analyzed for elicitation of humoral immune response and protective cancer immunity.

Conclusion : sFvs are useful tools to identify and isolate cell specific proteins and react to synthetic peptides which may be useful immunogens.

(iv) Determination of immunogenicity of isolated peptide mimotopes

Both peptides BTE6-LX-8b and BTE6X-15-7 elicited a distinct high titer humoral immune response with no cross-reactivity (Figure 21) when analyzed by ELISA assay in a solid support. However, when incubated in liquid media, some degree of cross raeactivity was observed as shown in Figure 22 for inhibition of binding of anti-BTEC-15-7 and for anti-BTEC-LX-8b (Figure 23).

In a vaccination study where the animals were vaccinated with 100 μg/ peptide on day 0 and 50 μg peptide/rat as booster on days 14, 21, and challenged with 10,000 MAT-LyLu cells on day 14, a very pronounced delay in tumor incidence, latency and rate of tumor growth

(Table 3 and Figure 24 and 25) was noted. Two of four animals vaccinated with the peptides were tumor free even after six weeks. This is a very significant result considering that 10,000 MAT-LyLu cells subcutaneously injected give rise to palpable tumors in ten days. These tumor specific reactivities may all be directed against the 170 kDa protein, since all these studies utilize scFv E6. The differences of gp96-peptide complexes and peptide in their anti-tumor effects may be reflective of antigen differences, dose and large number of antigens, associated with gp96, superior adjuvant property of gp96, or a cell mediated (for gp96) vs a humoral response (for the peptides), the peptides have a minimal disulfide constraints and thus only partially mimic gp96-peptide complexes. The ability of the synthetic peptides to bind human HLA (A and B)molecules (Web site SYFPEITHI, developed by Hans Georg Rammensee's lab) resulted in a matching score ranging from 12-28, where 30 is the highest HLA-peptide fit score.

Conclusion : Peptide mimotopes to tumor rejection antigen, gp96-peptide complexes can be identified and are strong candidates as cancer vaccines

v. Identification of peptides associated with gp96

Purified gp96-peptide complexes were acid digested e gp96 and peptides separated by Amicon filtration and analyzed using matric assisted laser desorption ionization mass spectrophotometry (MALDI-MS) peptide mass mapping analysis performed (Figures26-29). The sequence of some of the peptides is shown in Table 3. These data shows that indeed gp96-peptide complexes contain peptides that may be unique. Identification of these peptides by single chain antibodies is possible as demonstrated in experiments from Task 1. We however, extended our observation and wanted to demonstrate the utility of our analysis using a defined experimental model system which was designed as follows.

Purified tissue derived gp96 was reacted with a defined peptide called VSV-8 having the following sequence. Loaded and unloaded gp96 was differentially panned using the single chain combinatorial antibody library as detailed (Figure 30). Experimental results are shown in Figure 31 which demonstrate that specific antibodies that can react to only gp96-VSV8 peptide can be identified and isolated.

TASK 2 IS COMPLETE AS PROPOSED WITH ADDITIONAL DATA ON IDENTIFICATION AND CHARACTERIZATION OF SYNTHETIC PEPTIDE MIMICS

Completion of Tasks 1 and 2 allow us to make the following conclusions

- Synthetic scFv library can be used to differentially identify tumor associated antigens in prostate cancer
- These scFvs are useful tools to isolate cellular proteins and synthetic peptide mimotopes
- The synthetic peptide mimotopes can be used as tumor rejection antigens

- Heat shock protein, gp96, are useful source of tumor rejection antigens identifiable by single chain antibody library

Task 3: 24-30 months

Characterization of the distribution of the identified proteins in primary and metastatic prostate cancer lesions and in normal tissues of the proteins of origin of PAA derived peptides.

Both gp96 specific and cell surface specific single chain antibodies were examined for their ability to interact with cellular proteins by immuno-fluorescence and their ability to identify proteins by Western blotting. The results of the Western blot analysis is presented in Figure 32. These antibodies were found to be of low affinity with non-specific reactivity with several cellular proteins. The non-specific cellular reaction was presumably due to associated bacterial contaminants which is not an issue in immuno-precipitation studies but is an issue when the entire mammalian cell extract is subjected to gel electrophoresis.

The reactivity of single chain antibodies was found to be specific when reactivity was examined using immunofluorescence and MAT-LyLu and Dunning G cells (Figures 33, 34) . ScFv antibodies that were cell surface specific reacted well and in contrast scFvs that were specific to internal cellular peptides/proteins showed little or no reactivity.

TASK 3 HAS BEEN COMPLETED AS PROPOSED

Challenges encountered in Task 3

- The widespread use of single chain antibody in tissue screening by conventional western blotting and immuno-histochemistry needs further standardization
- Specificity of reaction of soluble single chain antibodies need to be validated with several non-reactive clones
- Large number of clones need to be screened to generate clones of high affinity and specificity

KEY RESEARCH ACCOMPLISHMENTS

- Tumor specific single chain antibodies (scFvs) reactive to either gp96-peptide complexes or to cell surface can be isolated and their reactivity is specific. Our panning strategy gave adequate number of scFvs for a comprehensive analysis.
- sFvs are useful tools to identify and isolate cell specific proteins and react to synthetic peptides which may be useful immunogens.
- Peptide mimotopes to tumor rejection antigen, gp96-peptide complexes can be identified and are strong candidates as cancer vaccines
- Cellular distribution of protein/peptides can be examined using single chain antibodies and immuno-fluorescence

REPORTABLE OUTCOMES

1. Yedavelli SPK, Guo L, Daou ME, Srivastava PK, Mittelman A, Tiwari R.K. Preventive and therapeutic effect of tumor derived heat shock protein, gp96, in an experimental prostate cancer model. Int J Mol Med. 4: 243-248, 1999.

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13. Invention disclosure : Cancer vaccine development using novel antigens, tumor rejection antigen.

14. Invention disclosure : Tumor antigen mimotopes

CONCLUSIONS

- We have successfully used combinatorial single chain phage display library (scFv) for the detection of tumor rejection antigens and to define the heterogeneity of cancer antigens in prostate cancer.

- Most importantly we were able to identify synthetic peptide mimotopes to tumor rejection antigen gp96 and in the future used as standardized immunogens to prevent metastases.

- We also challenged an existing paradigm that T cell (cell mediated) and B cell (humoral) defined epitopes are entirely distinct and there are no overlaps. Our results (and results of several other studies) now confirm that T cell defined epitopes can be recognized by antibodies and that there may exist considerable overlap. In this respect, the existing paradigm was challenged.

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1. Yedavelli SPK, Guo L, Daou ME, Srivastava PK, Mittelman A, Tiwari RK. Preventive and therapeutic effect of tumor derived heat shock protein, gp96, in an experimental prostate cancer model. *Int J Mol. Med. 4:243-248, 1999.*
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List of Personnel receiving pay from the research effort in this grant.

1. Dr. Raj K Tiwari
2. Dr. Ashok Badithe
3. Dr. Srirupa Mukhopadhyay
4. Dr. Yuangen Chen

Figure 1

CONSTRUCTION OF PHAGE DISPLAY LIBRARY

Tiwari, Raj K.

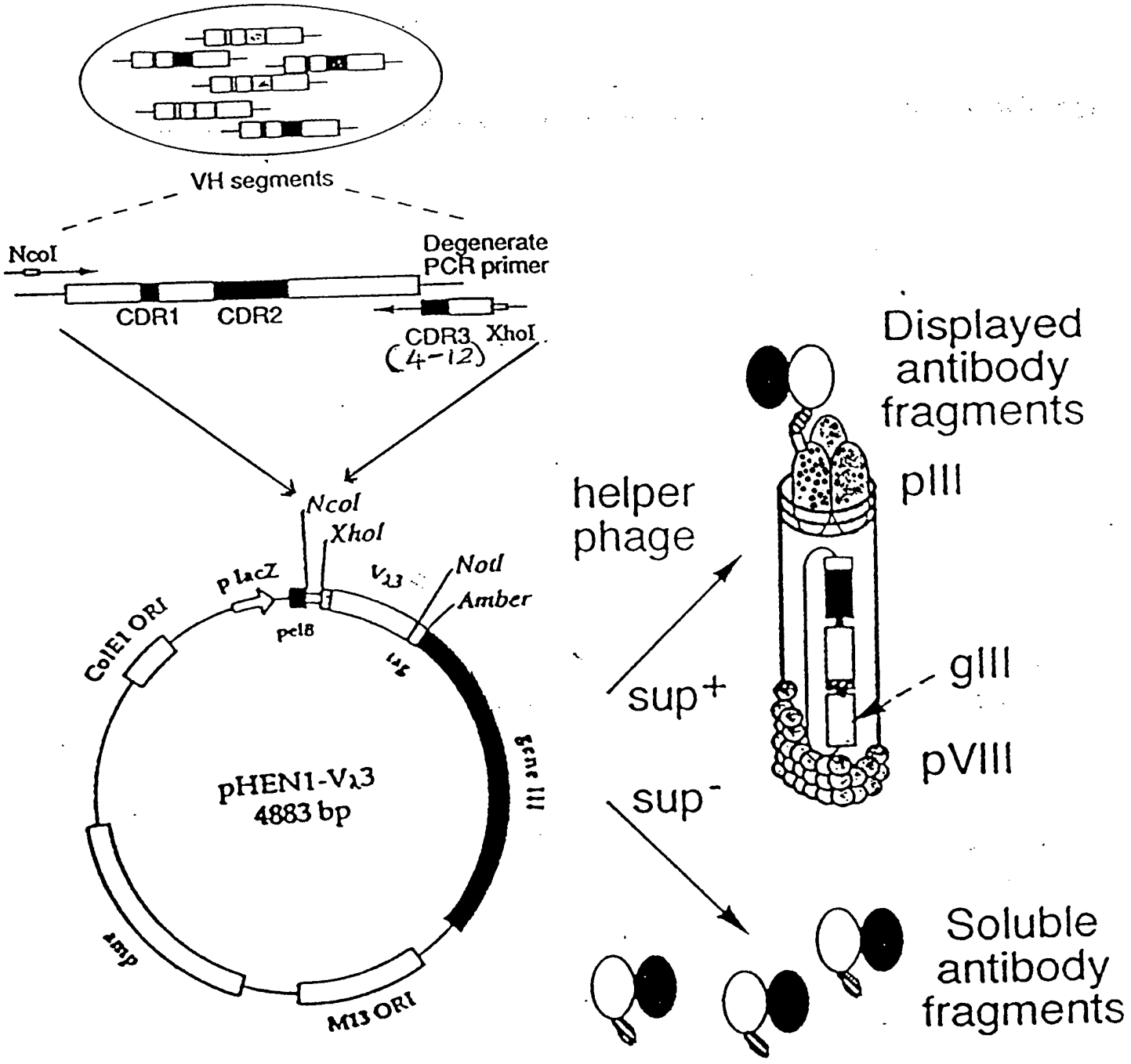


Fig. 2. PANNING STRATEGY FOR gp96-PEPTIDE COMPLEXES

Library of single chain phage display combinatorial antibodies panned over normal liver derived gp96-peptide complexes

↓

Unabsorbed phages panned again over liver derived gp96-peptides complexes - 3X

↓

Unabsorbed phages panned over Mat-Ly-Lu (tumor) derived gp96-peptide complexes

↓

Absorbed phages eluted and amplified

↓

Amplified phages panned again over liver derived gp96-peptide complexes

↓

Unabsorbed phages panned over tumor derived gp96-peptide complexes

↓

Absorbed phages eluted and amplified for three rounds of differential panning between liver and tumor derived gp96-peptide complexes

↓

Absorbed phages at the last stage of panning eluted and tested for reactivity to gp96-peptide complexes by ELISA

Fig. 3. PANNING STRATEGY FOR CELL SURFACE ANTIGENS

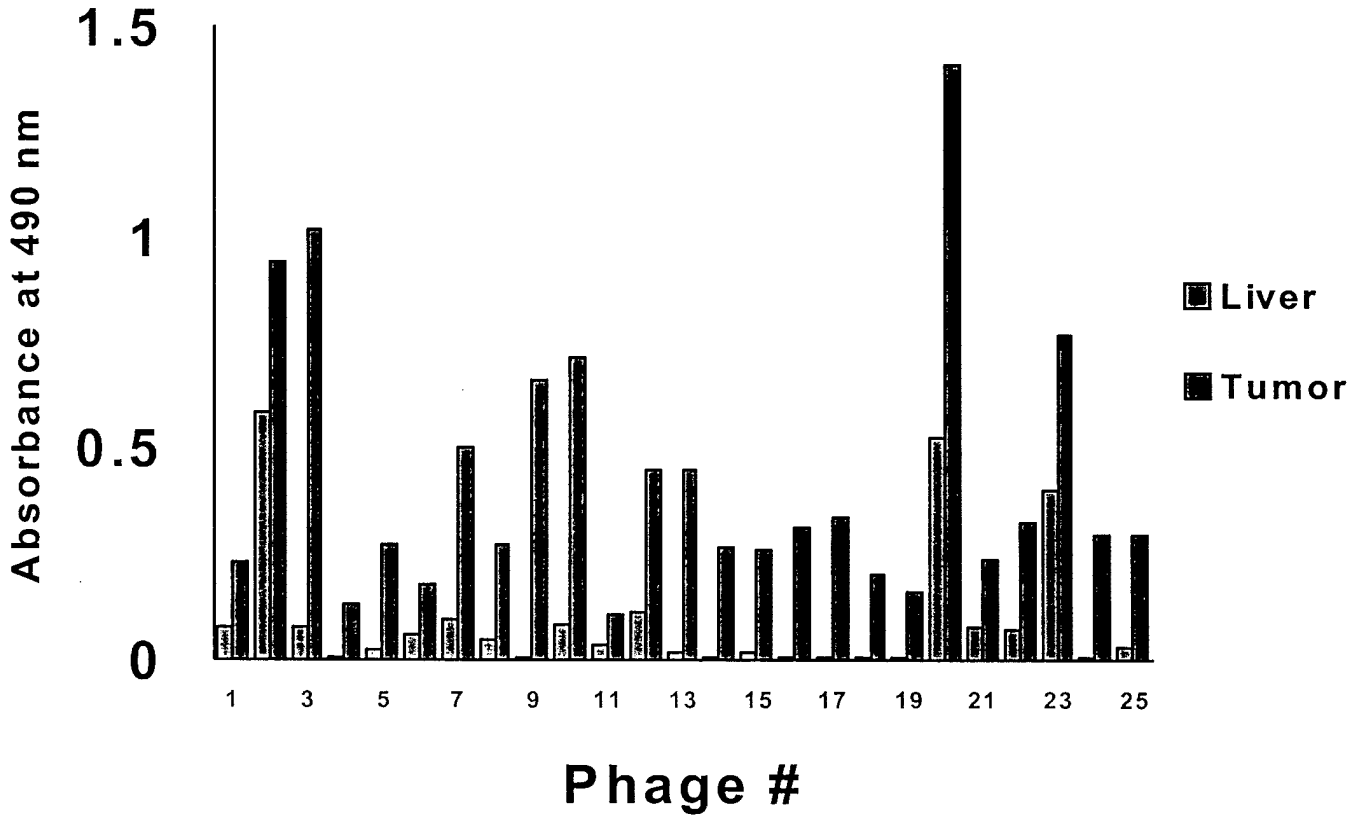
Library of single chain phage display combinatorial antibodies panned over Mat-Ly-Lu (MLL) cells

Bound phages were eluted and amplified

The phages were subjected to two more rounds of panning over MLL cells and bound phages were eluted and amplified each time

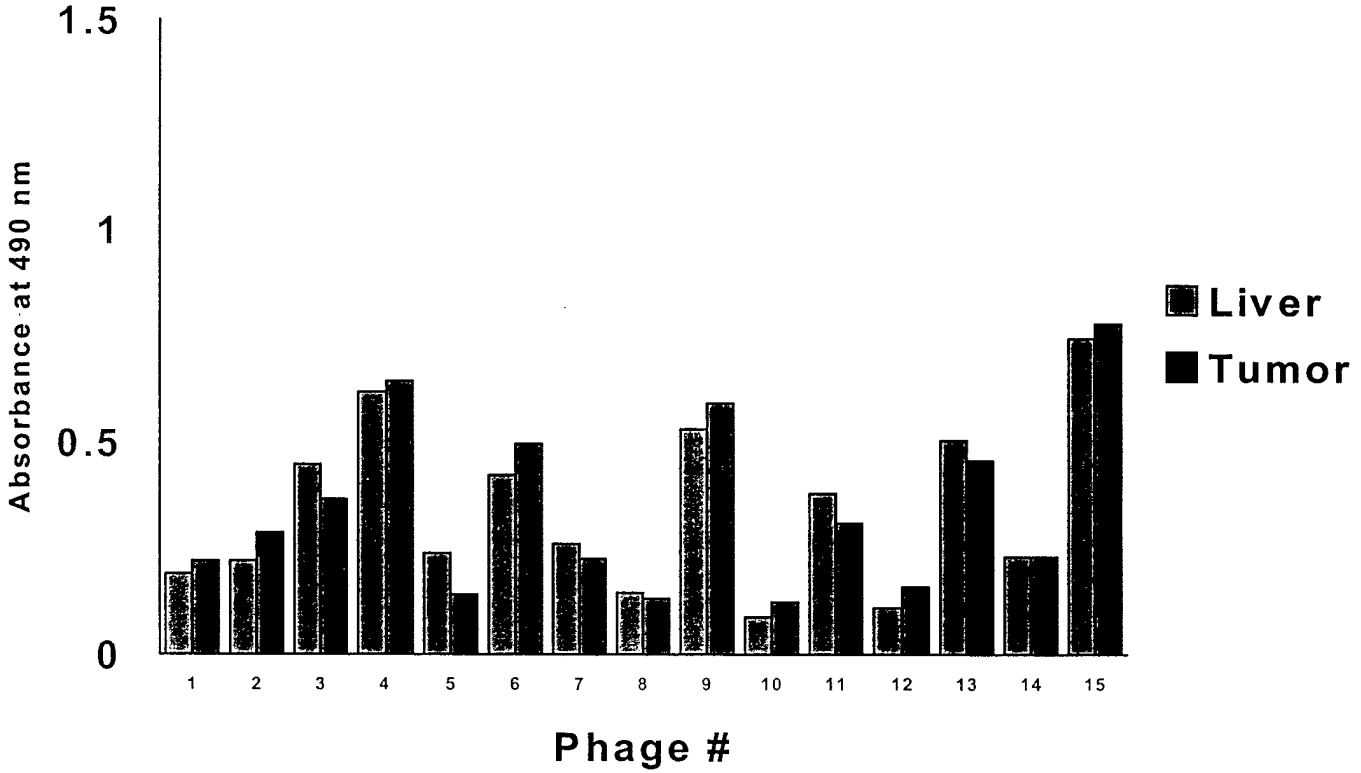
After the last round of panning, single phages were isolated and tested for reactivity to MLL, DG and TSU prostate cancer cell lines by ELISA

Fig. 4. Phage clones specific for tumor derived gp96-peptide complexes



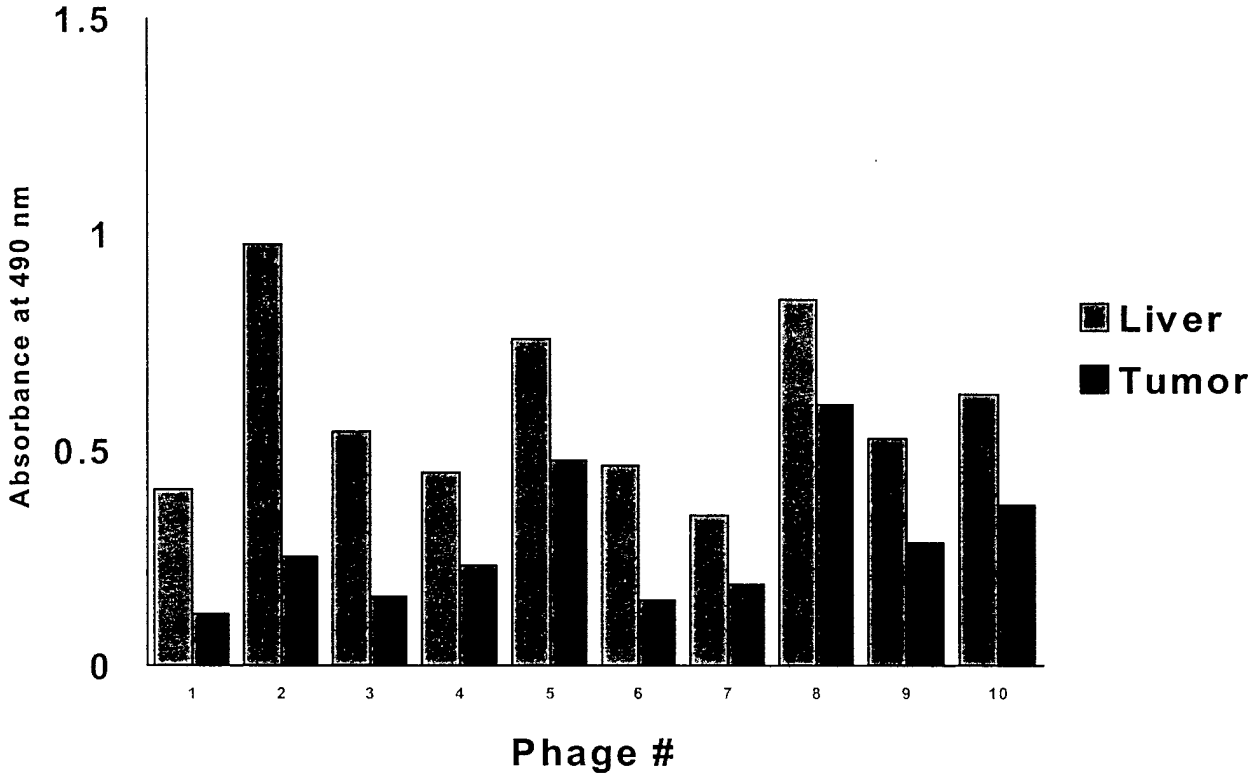
Tumors were induced by MAT-LyLu in Copenhagen rats
Livers for gp96 extraction were tumor free and derived from the same animal

Fig. 5. Phage clones that react with both liver and tumor derived gp96



Tumors were induced by MAT-LyLu in Copenhagen rats
Livers for gp96 extraction were tumor free and derived from the same animal

Fig. 6. Phage clones that have higher reactivity with liver derived gp96



Tumors were induced by MAT-LyLu in Copenhagen rats
Livers for gp96 extraction were tumor free and derived from the same animal

Fig. 7. Reactivity of scFv fragments with gp96-peptide complexes (ELISA)

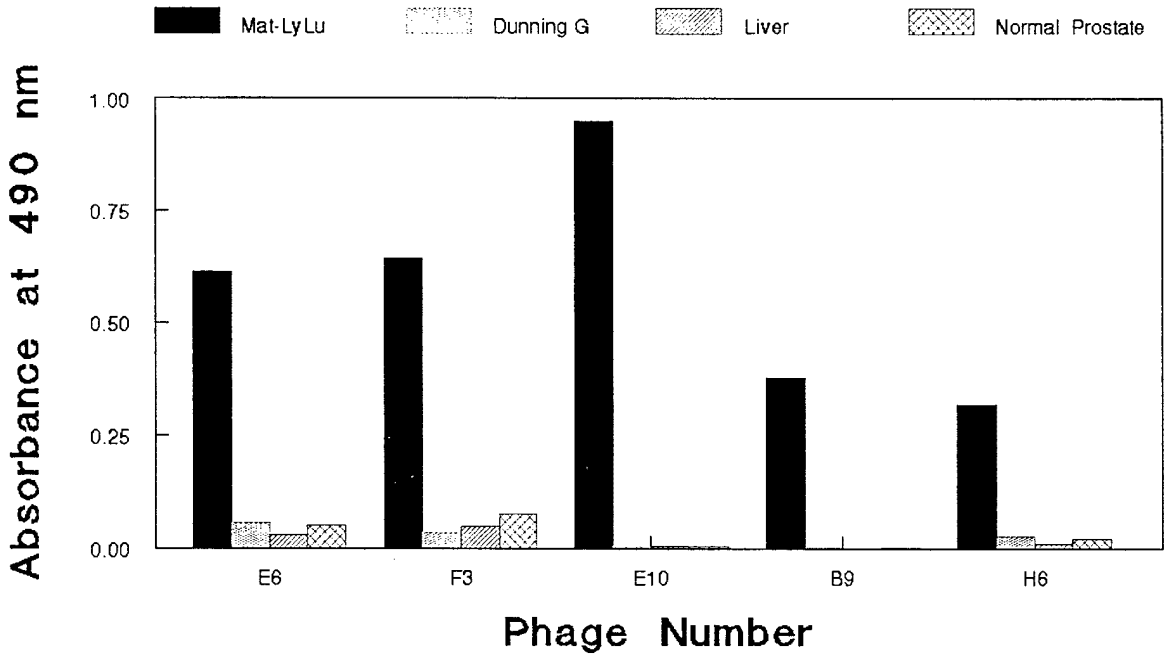


Fig. 8. Reactivity of tumor derived gp96 specific scFv E6 with intact prostate cancer cells

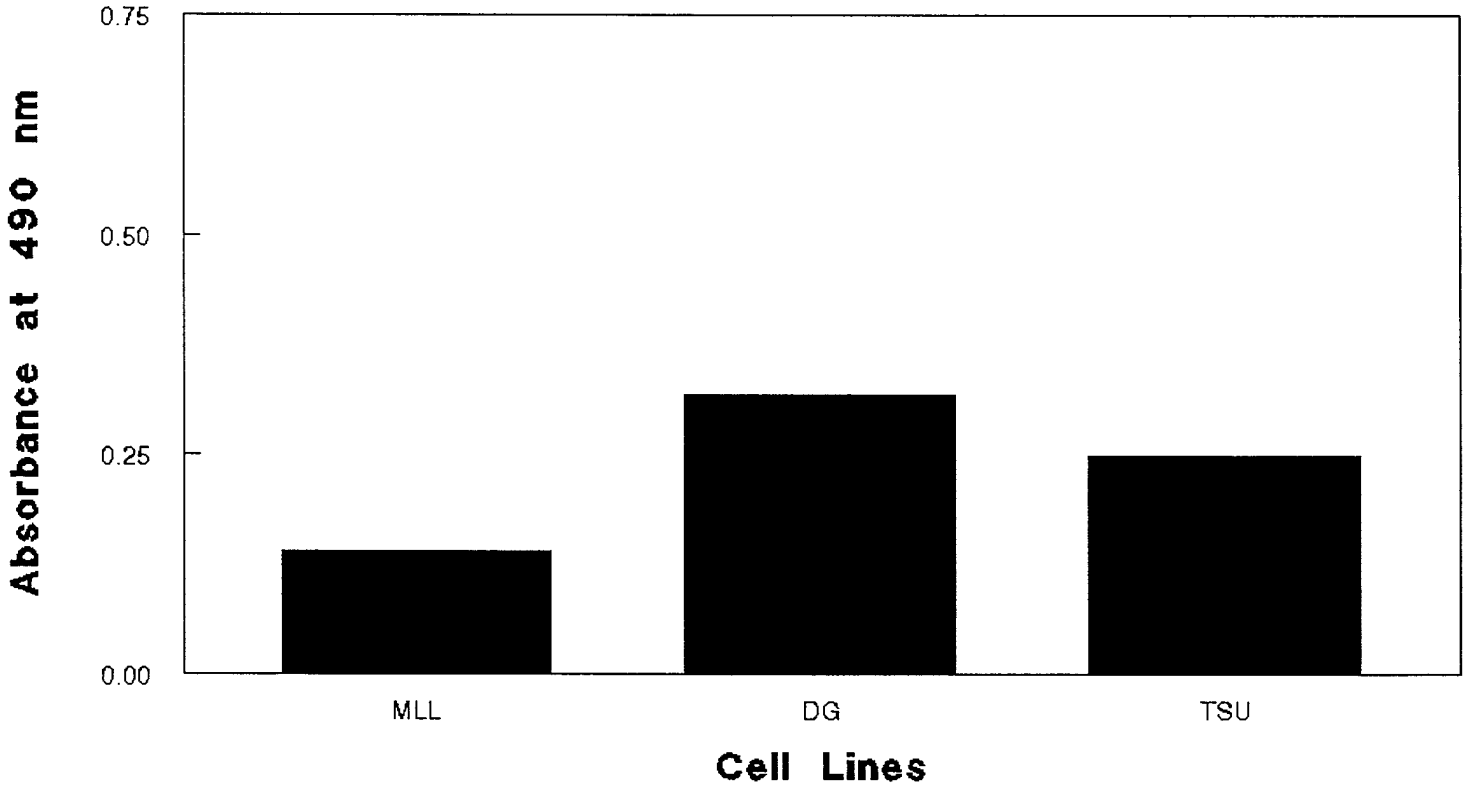


Fig. 9. Differential reactivities of scFvs to cell surface metastatic (MLL) and non-metastatic (DG) proteins and gp96-peptide complex

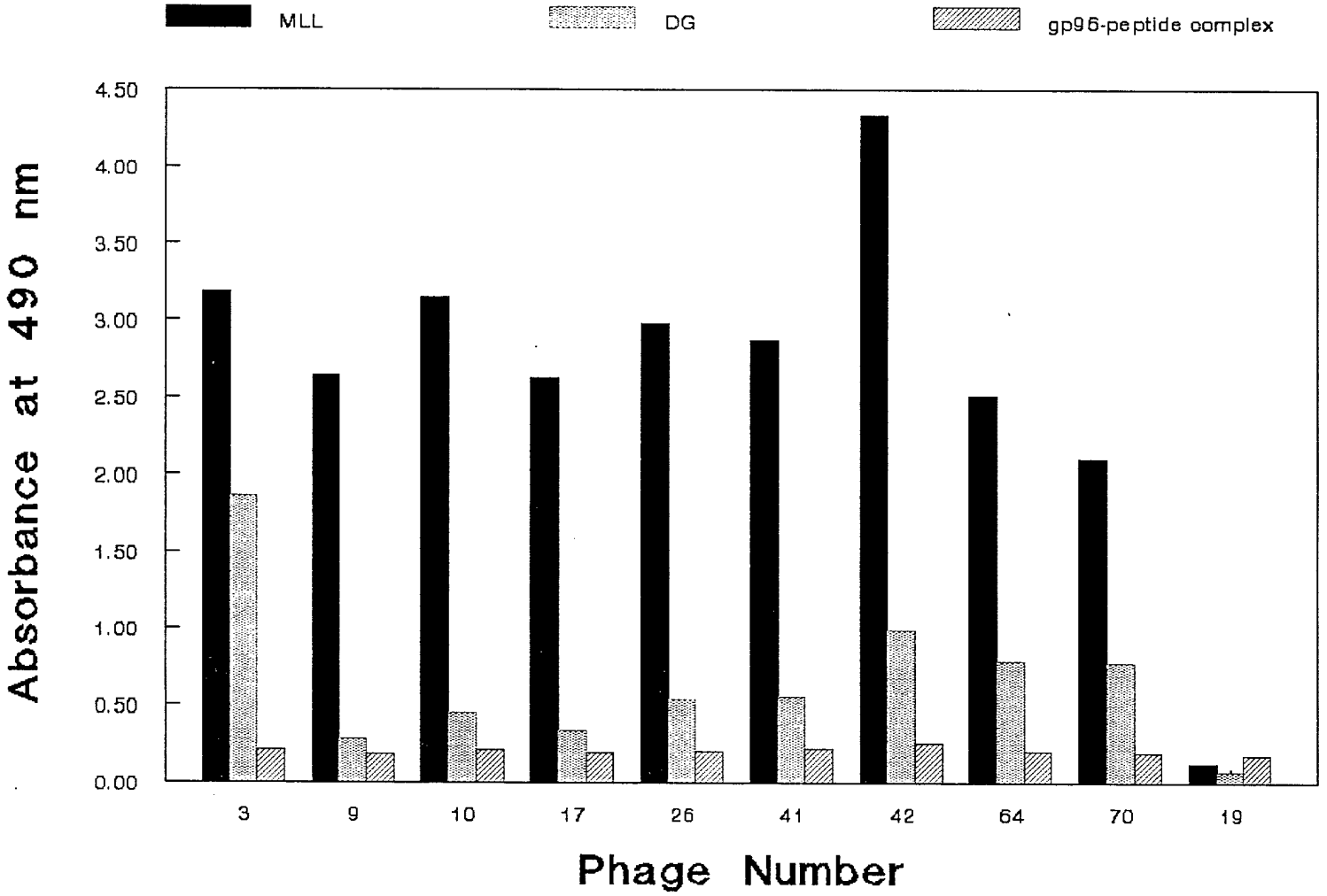


Fig. 10. Reactivity of scFvs with TSU cells

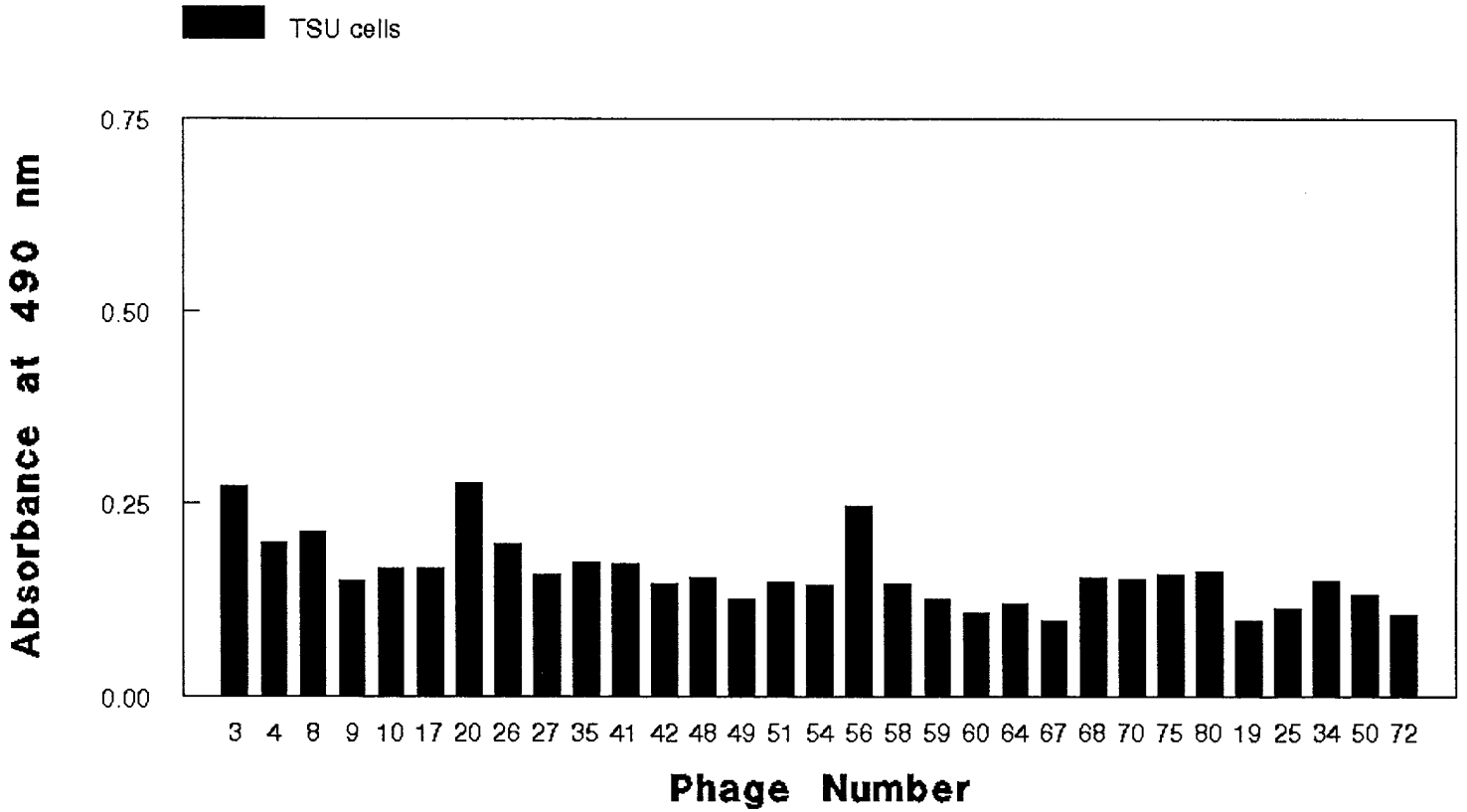


Fig. 11. scFvs (E6/F3) react with a 170 kDa MAT-LyLu specific protein detected by ^{35}S-Met metabolic labeling

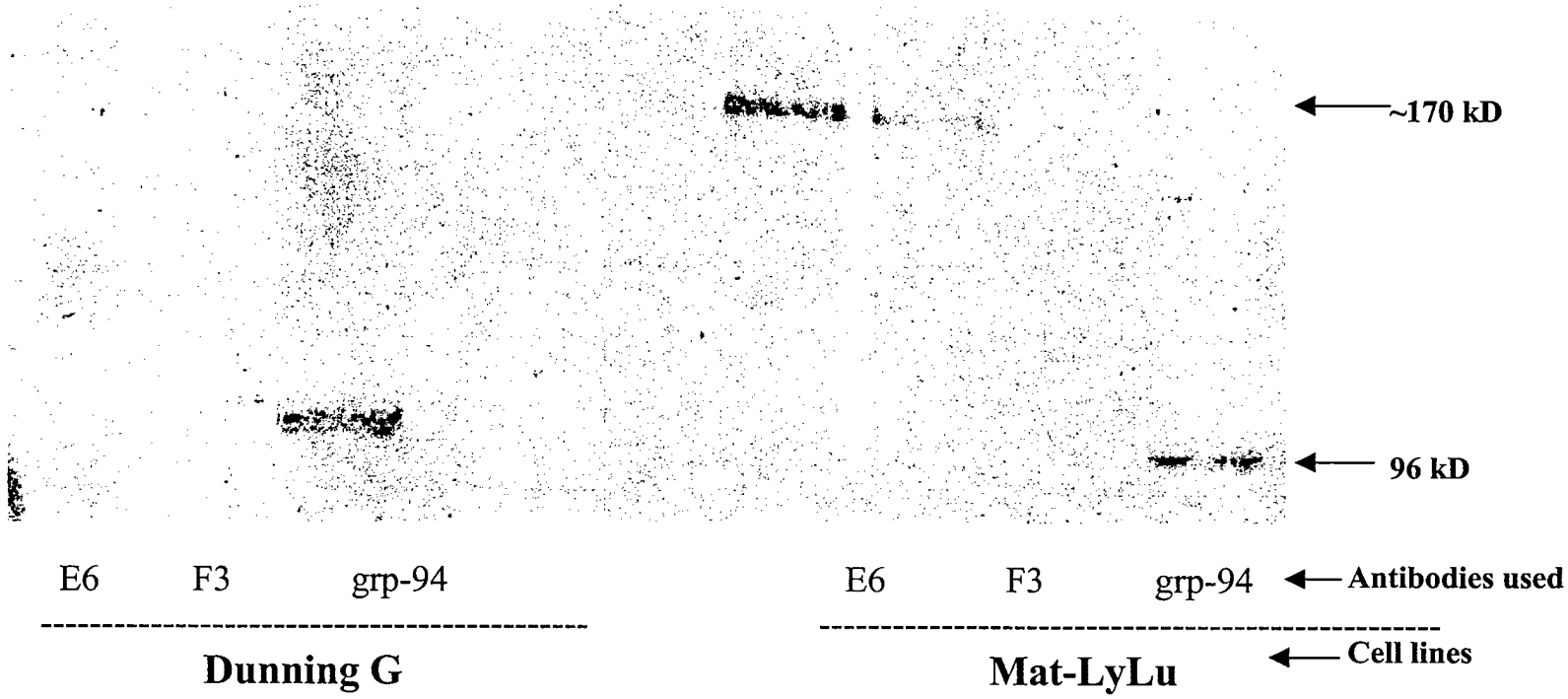


Fig. 12. scFv E6 immunoprecipitates a 170 kD protein in MAT-LyLu cells (detected by reversible E-Zinc stain)

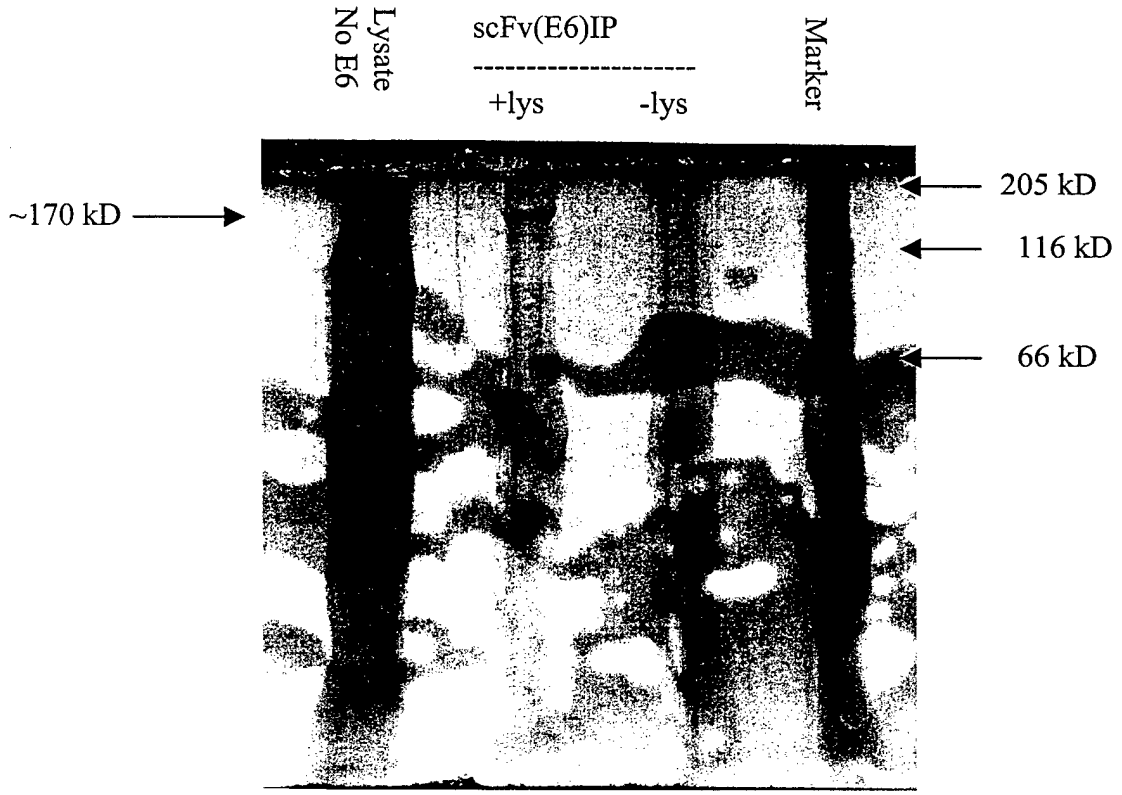
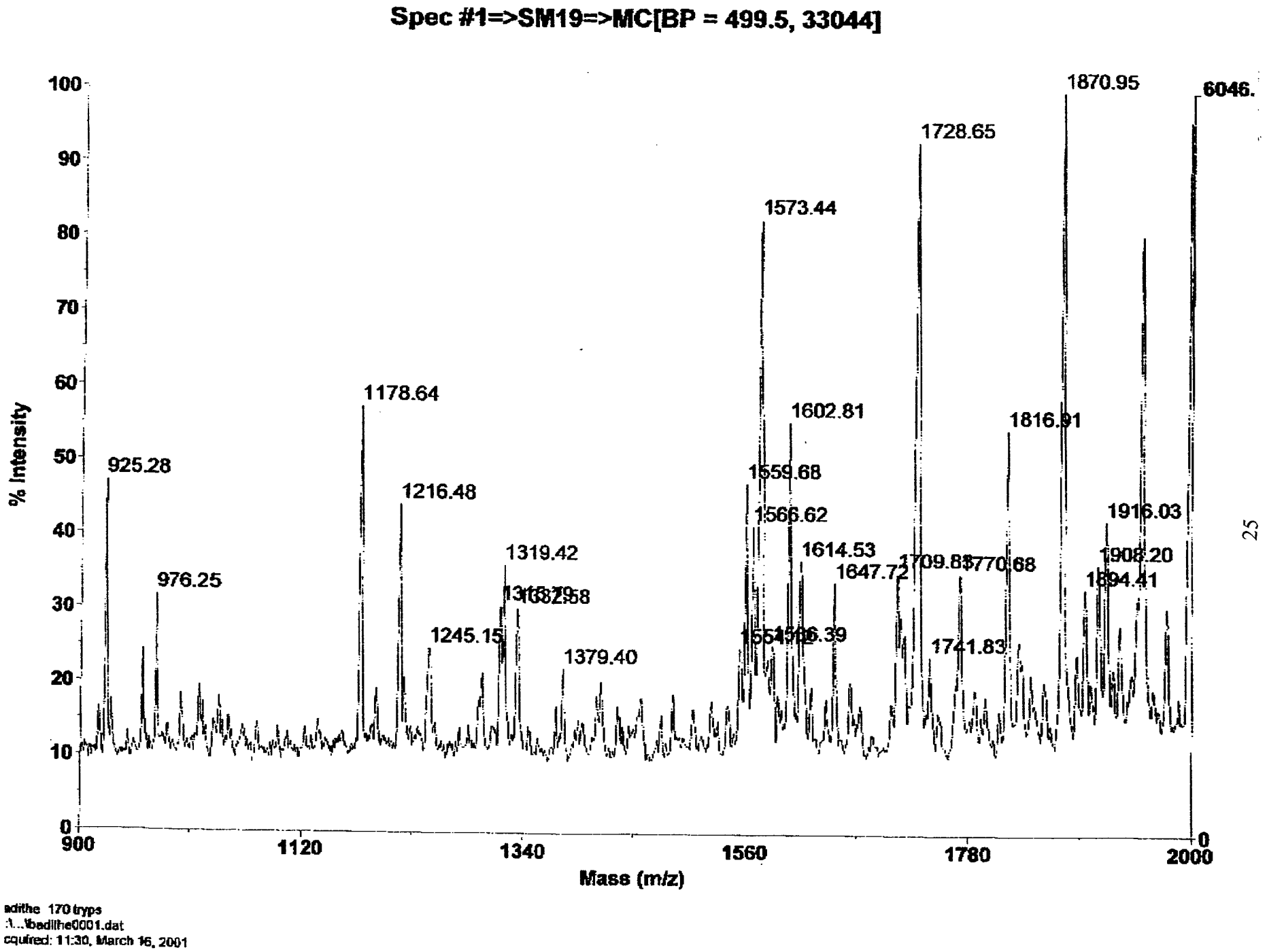


Figure 13



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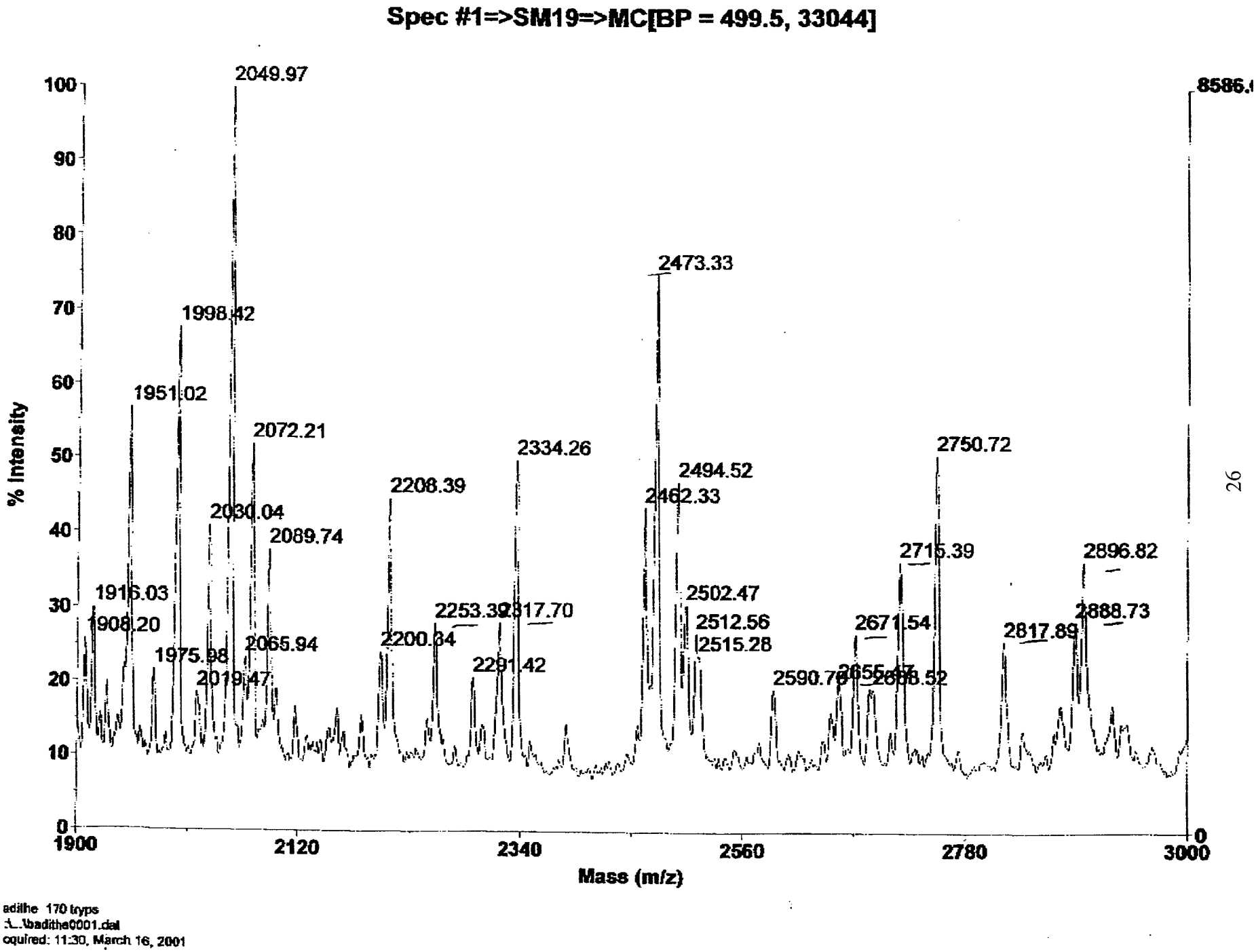
cquired: 11:30, March 16, 2001

Figure 14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2030.04
2089.74
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2334.26
2473.33
2494.52
2462.33
2750.72
2715.39
2896.82
2502.47
2512.56
2515.28
2888.73
2671.54
2817.89
2253.39
2317.70
2200.34
2291.42
2065.94
2019.47
1975.98
1908.20
1916.03
2590.76
2655.47
2698.52
8586.4
100
90
80
70
60
50
40
30
20
10
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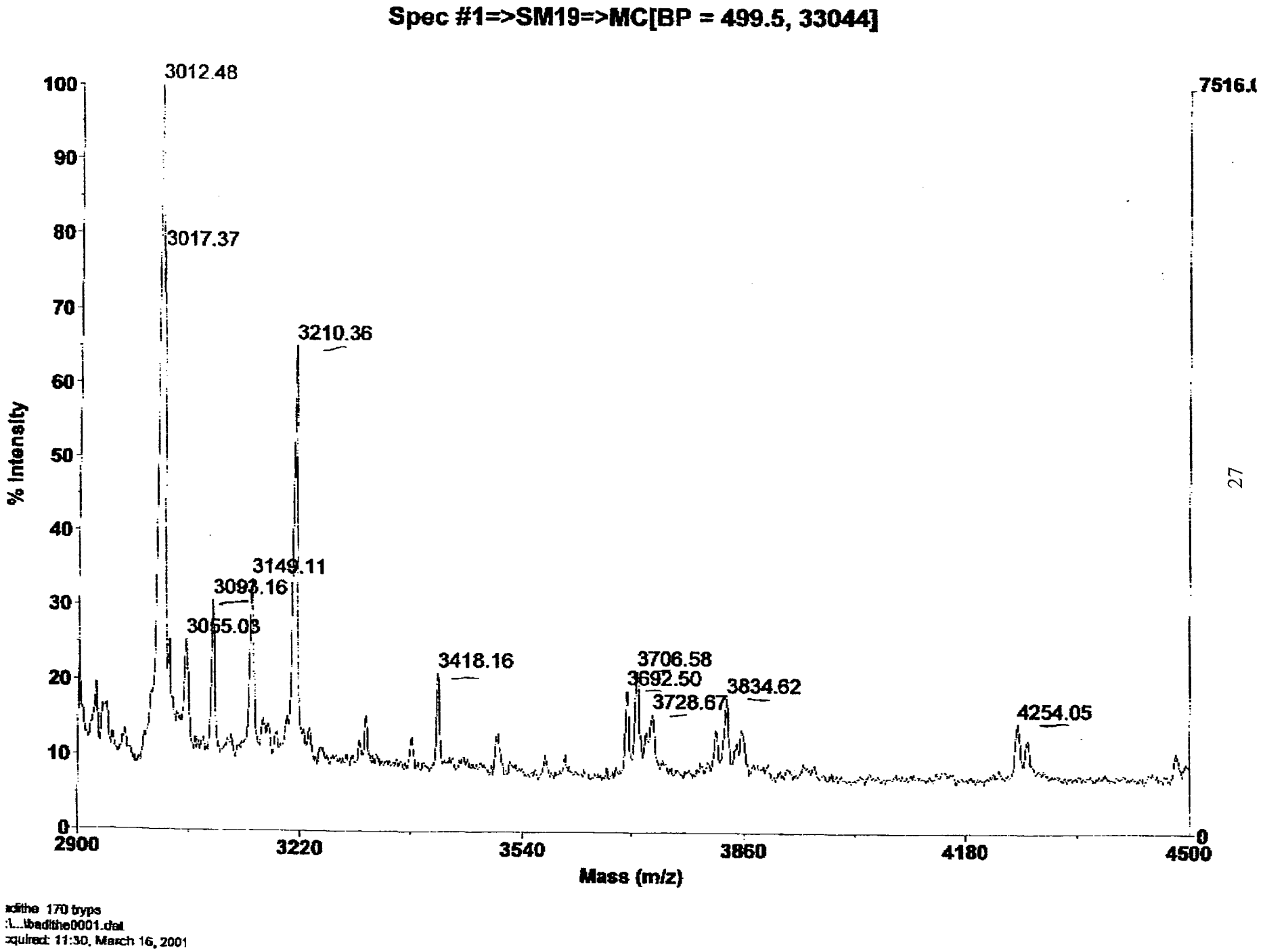
oquired: 11:30, March 16, 2001

Figure 15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3017.37
3210.36
3149.11
3093.16
3055.03
3418.16
3706.58
3692.50
3728.67
3834.62
4254.05
7516.(
% Intensity
Mass (m/z)
100
90
80
70
60
50
40
30
20
10
0
2900
3220
3540
3860
4180
4500
adithe 170 typs
:\...\badithe0001.dat
Acquired: 11:30, March 16, 2001

Fig. 16. PANNING STRATEGY FOR SELECTING PEPTIDE MIMICS

Phage display peptide libraries LX8 (12-mer containing disulfide bridge) and X15 (linear 15-mer) were panned over E6, a MAT-LyLu specific scFv, to affinity select peptides binding to the antibody

↓

Biotinylated E6 (1 µg in 35 µl TBS) was coated to streptavidin coated 96 well microtiter plates for 1 hr at 4°C in a humidified box

↓

Antibody was washed and wells were blocked with 300 µl blotto (5% milk, 10 mM EDTA) containing 0.12 mM biotin at room temperature for 2 hr

↓

Added 50 µl blotto and 100 µl TBS containing 10^{12} virions to the well and incubated at 4°C in a humidified box for 4 hr

↓

Wells were washed and bound phages eluted by adding 35 µl of elution buffer (0.1 M HCl, pH 2.2) and incubating at RT for 10 min

↓

Eluted phages were neutralized with 6.6 µl of 1 M Tris, pH 9.1 and then infected into *E coli* K91 strain and amplified for further rounds of panning

↓

Four rounds of panning were performed to select, enrich and amplify specific phages

↓

Specificity of phages tested by immunoscreening and ELISA

↓

Phage clones purified, DNA sequenced and peptide sequences obtained

Fig. 17. Reactivity of phages of peptide library to soluble single chain Fv antibody E6 by immunoscreening

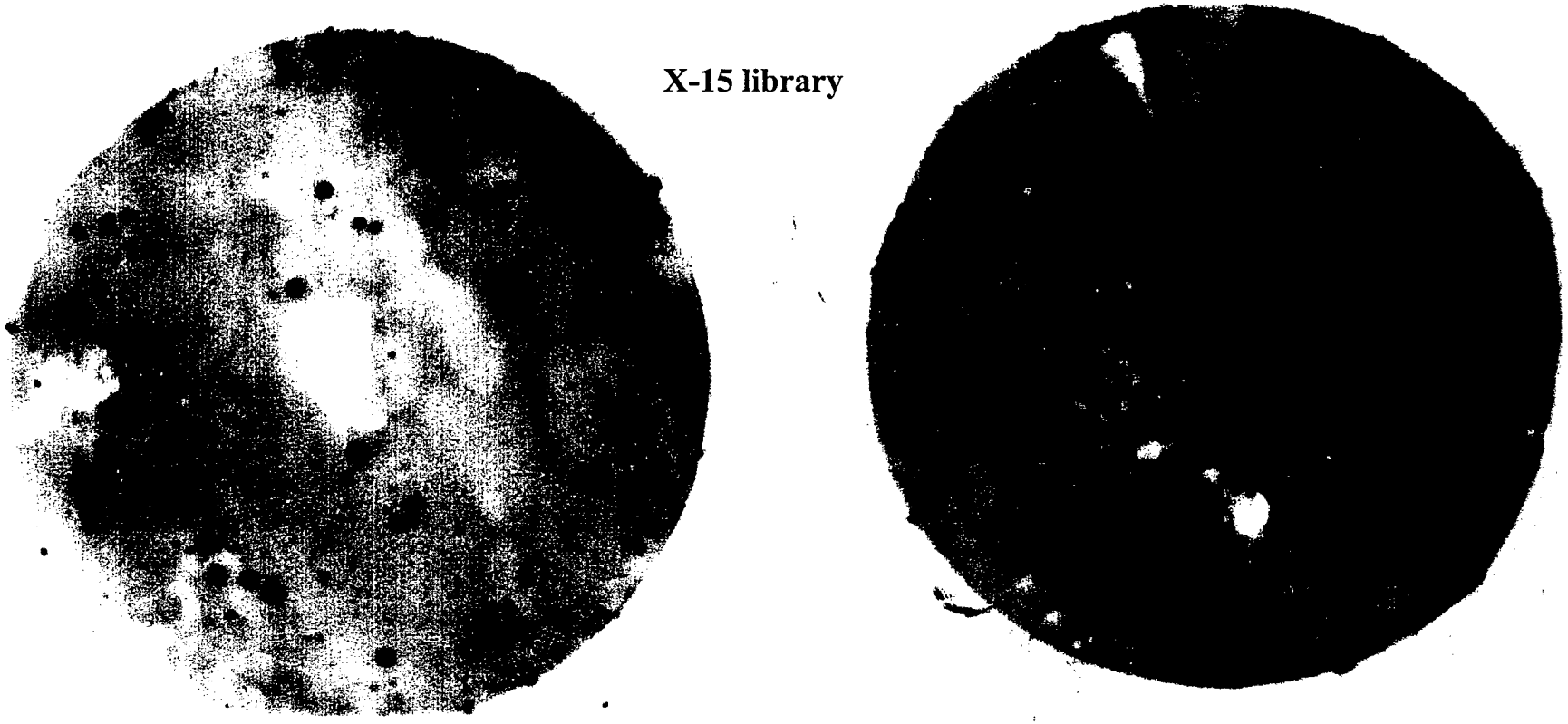
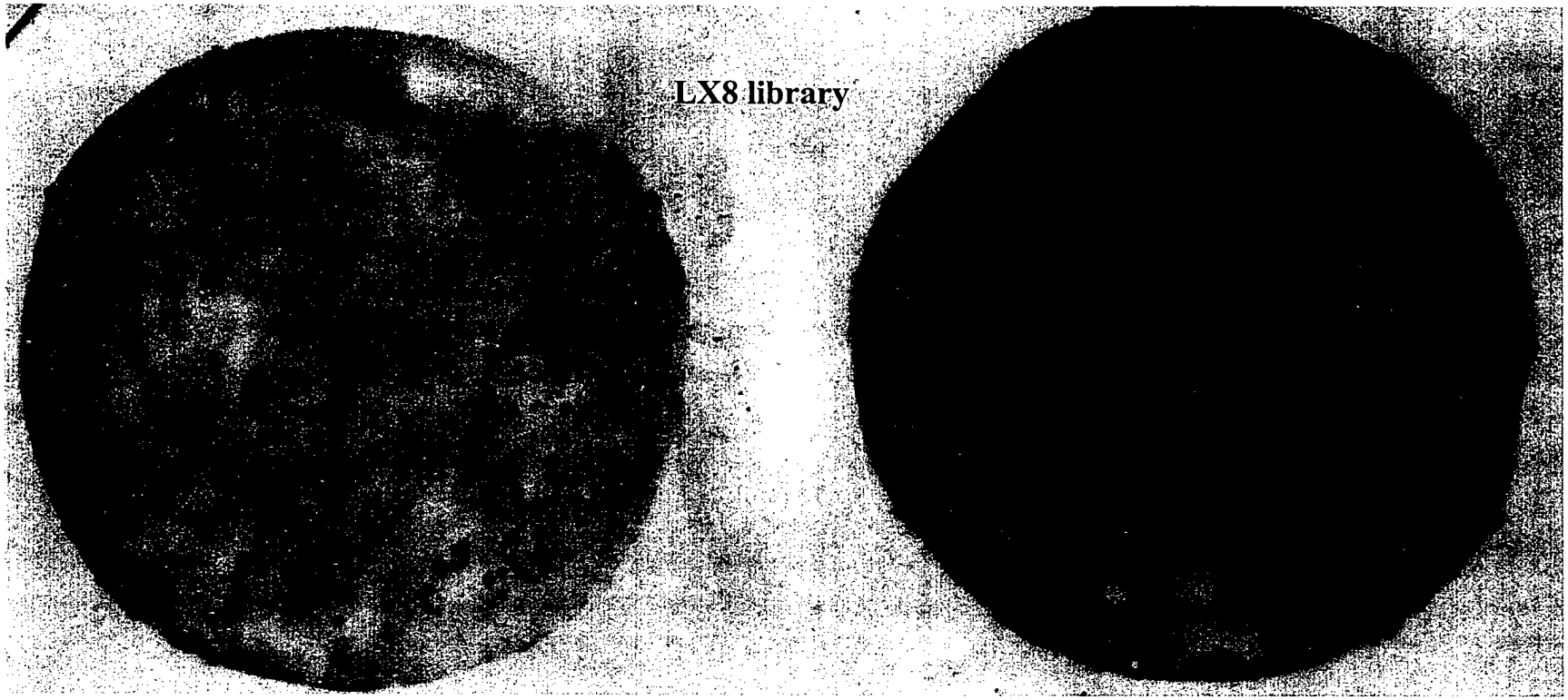


Fig. 18. Reactivity of phages of peptide library to soluble single chain Fv antibody E6 by immunoscreening



E6

Negative control scFv B11

Fig. 19. Reactivity of peptide phages of X-15 library to scFv E6

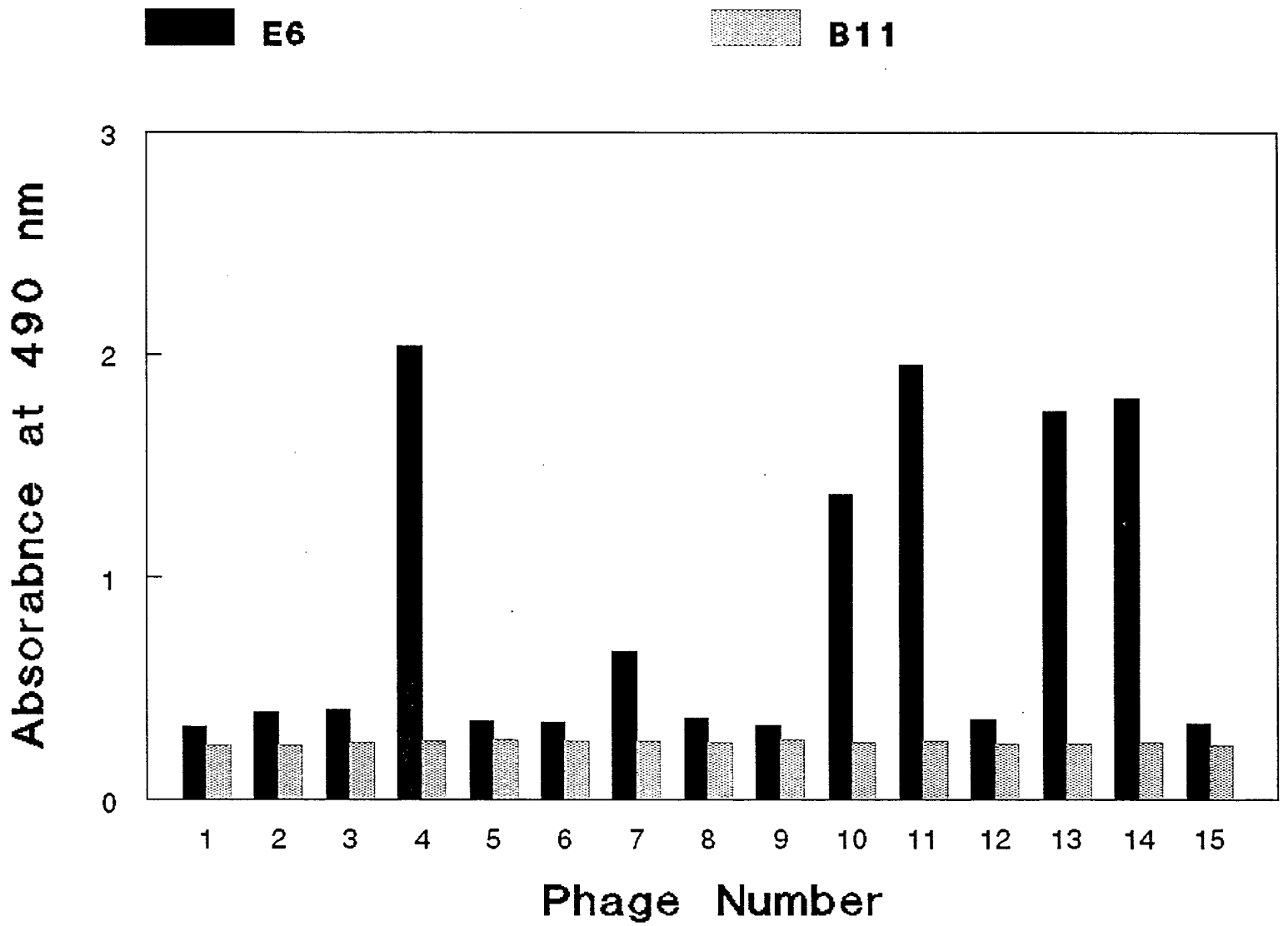


Fig. 20. Reactivity of peptide phages of LX-8 library to scFv E6

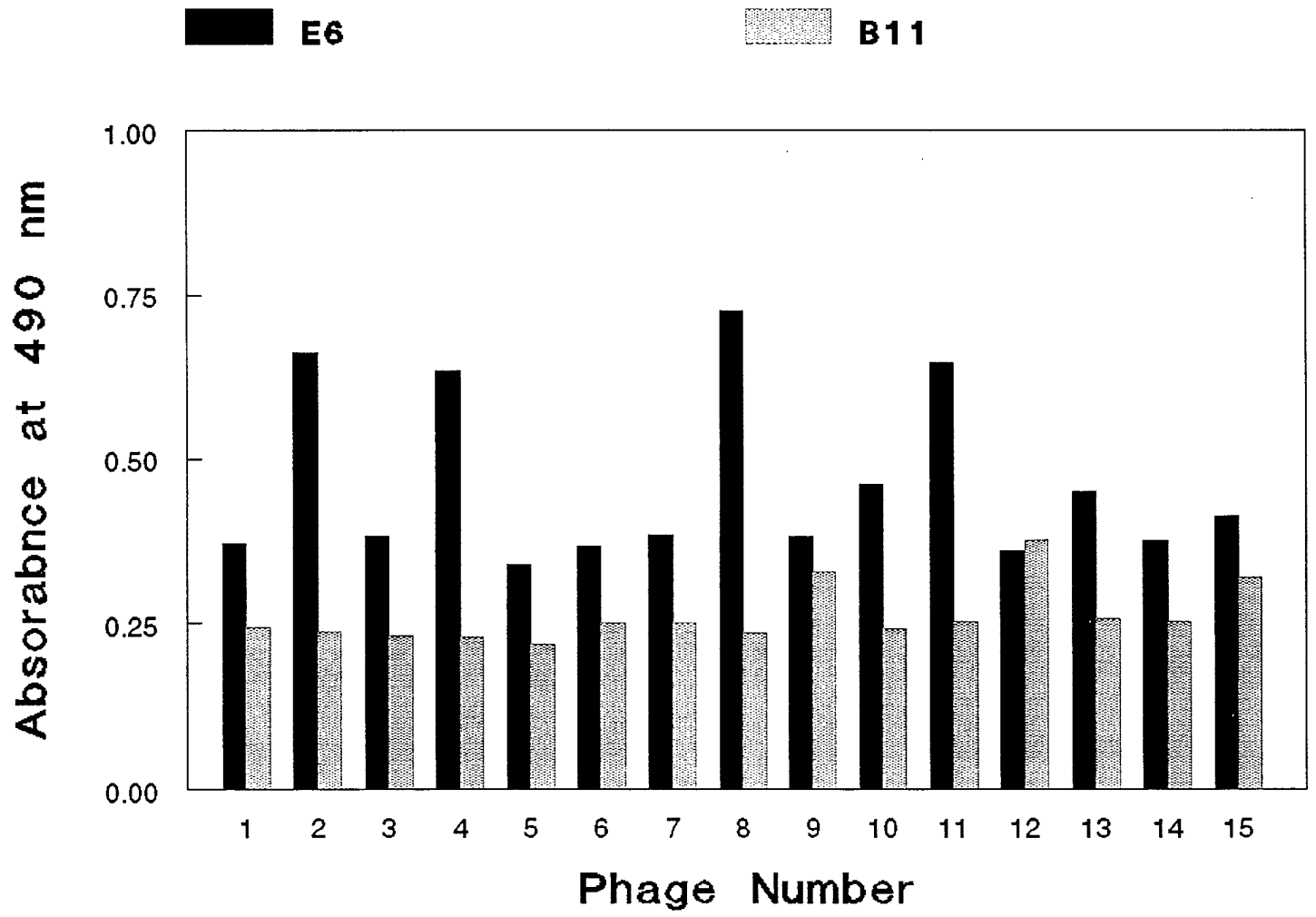


Fig. 21. Immune reactivity of antisera to peptides

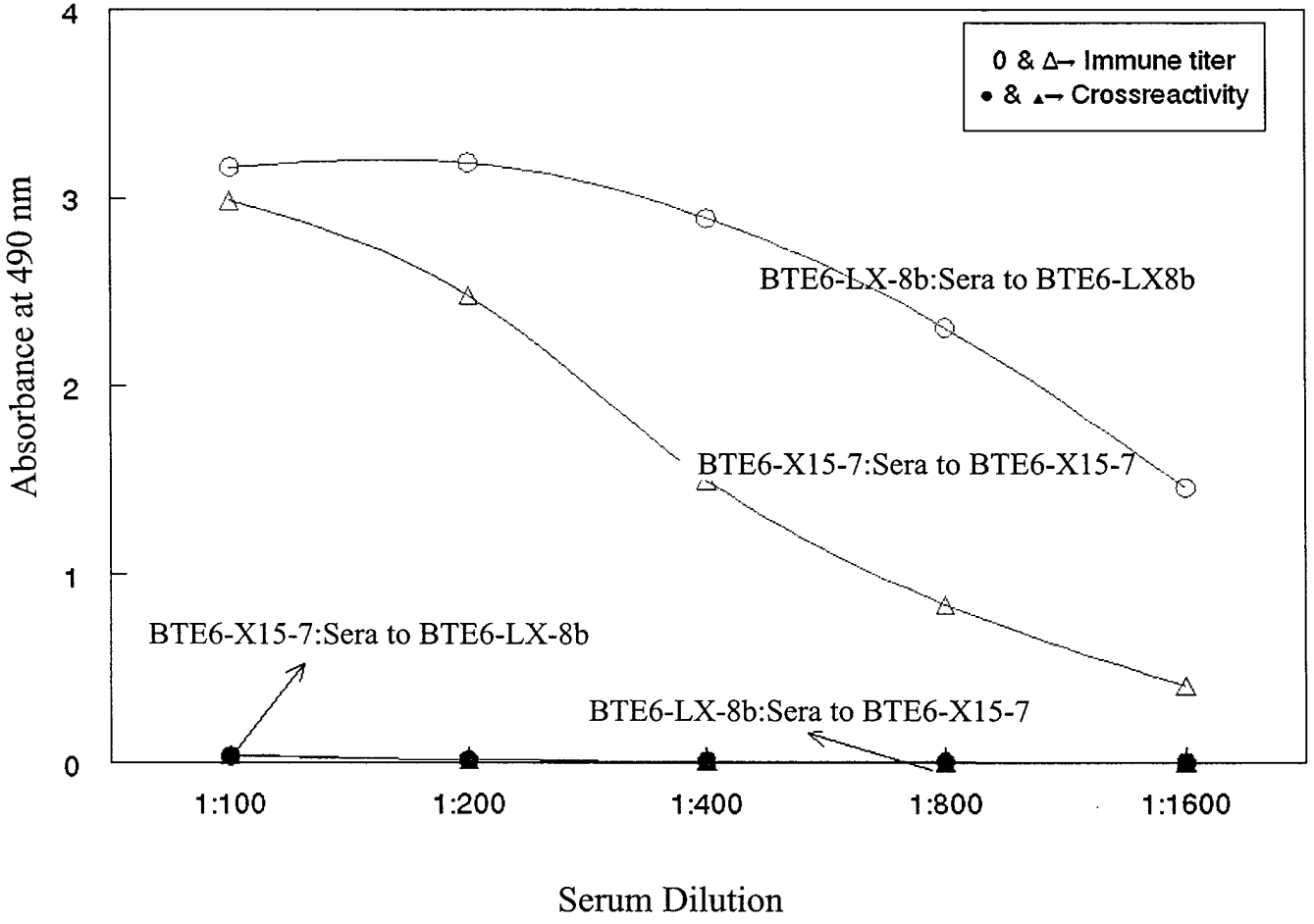


Fig. 22. Inhibition of binding of anti-BTE6-X15-7 antibodies to BTE6-X15-7 by BTE6-X15-7 and BTE6-LX8b

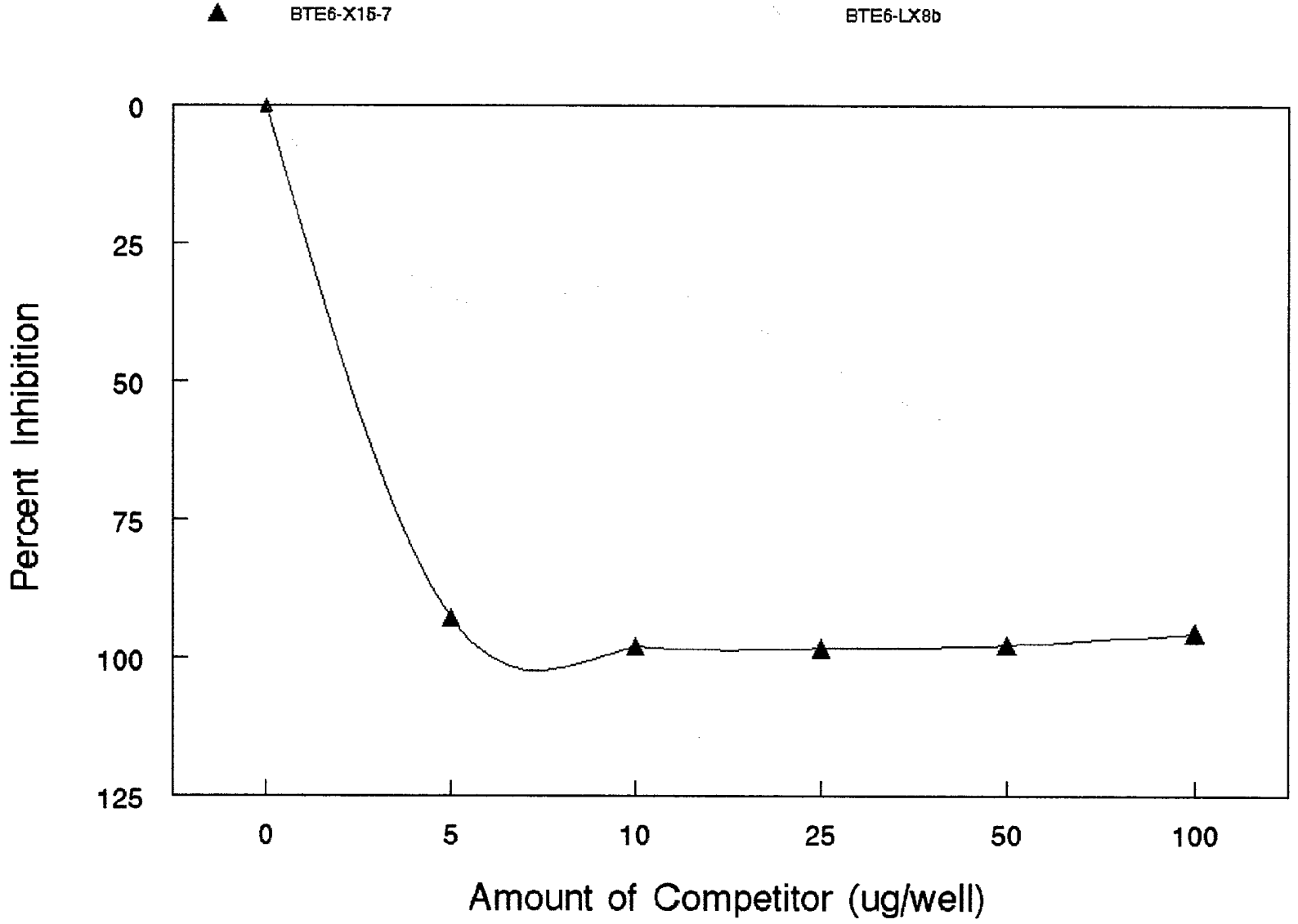


Fig. 23. Inhibition of binding of anti-BTE6-LX-8b antibodies to BTE6-LX-8b by BTE6-X15-7 and BTE6-LX8b

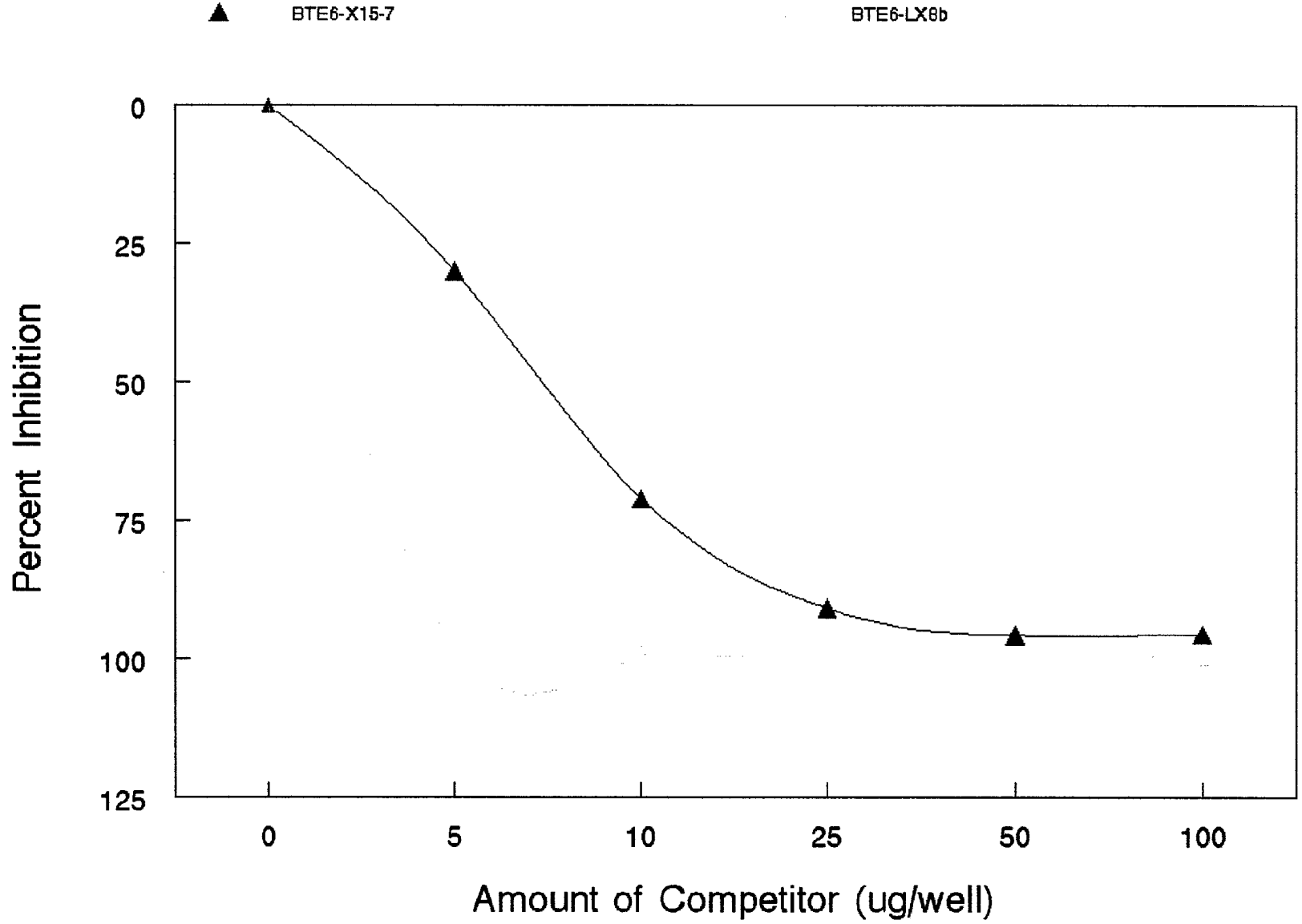


Fig. 24. Effect of peptide vaccination on rate of MLL induced tumor growth

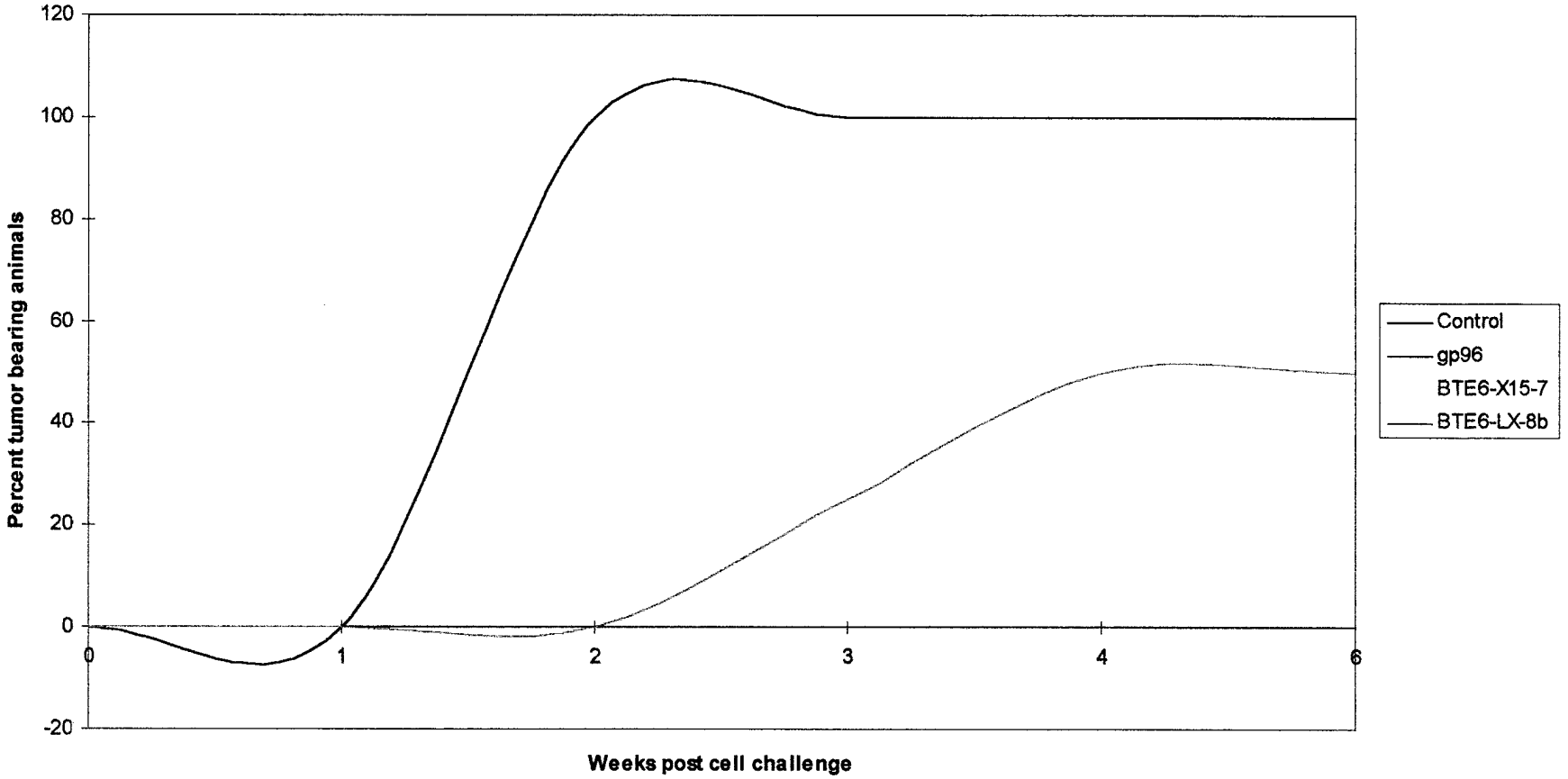
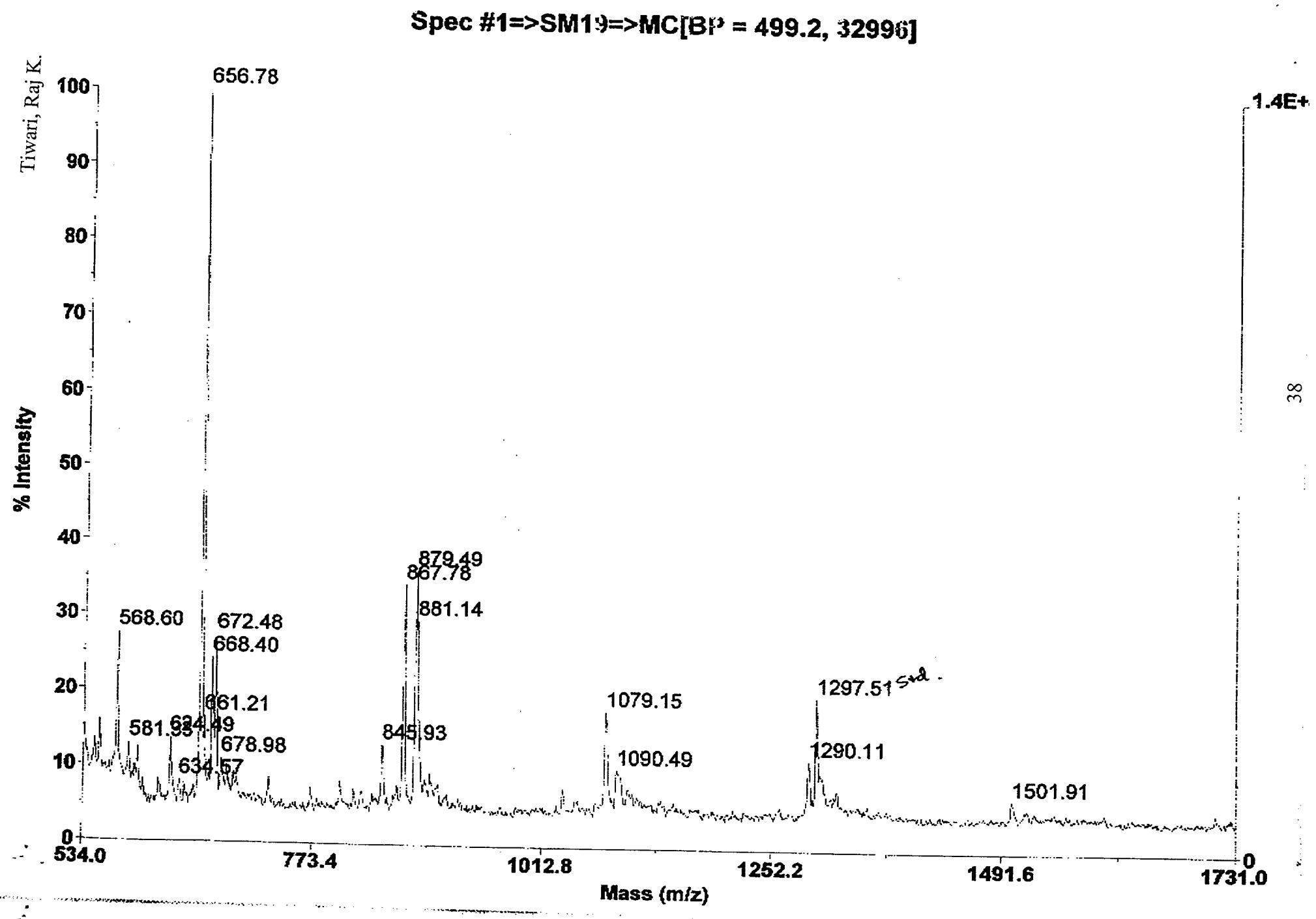


Fig. 25. Effect of vaccination of synthetic peptides on MAT-LyLu tumor size after 2 weeks

FIGURE 26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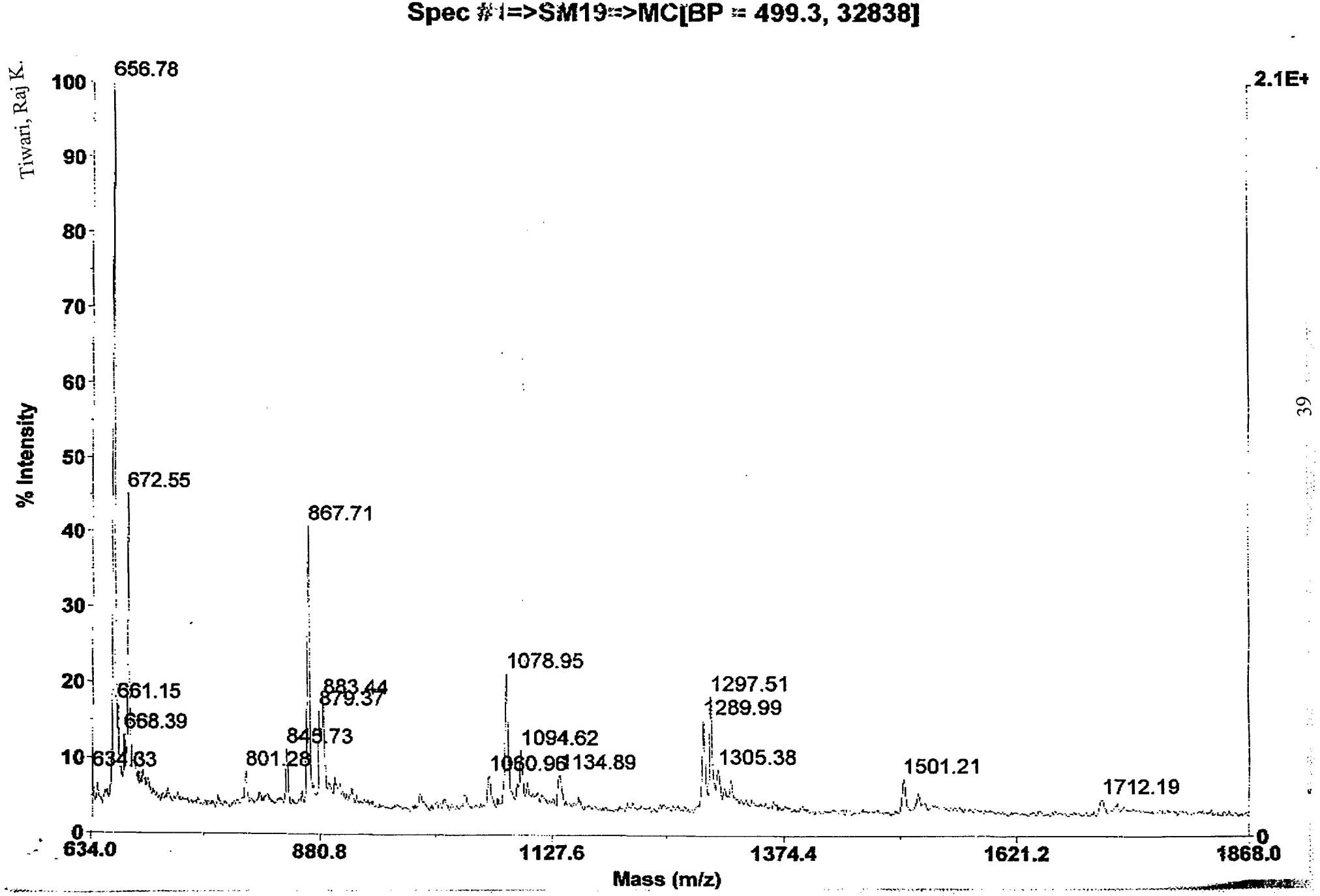
80
70
60
50
40
30
20
10
0
879.49
867.78
881.14
568.60
672.48
668.40
661.21
581.93
624.49
678.98
634.57
845.93
1079.15
1297.51
1090.49
1290.11
1501.91
534.0
773.4
1012.8
1252.2
1491.6
1731.0
Mass (m/z)
38

FIGURE 27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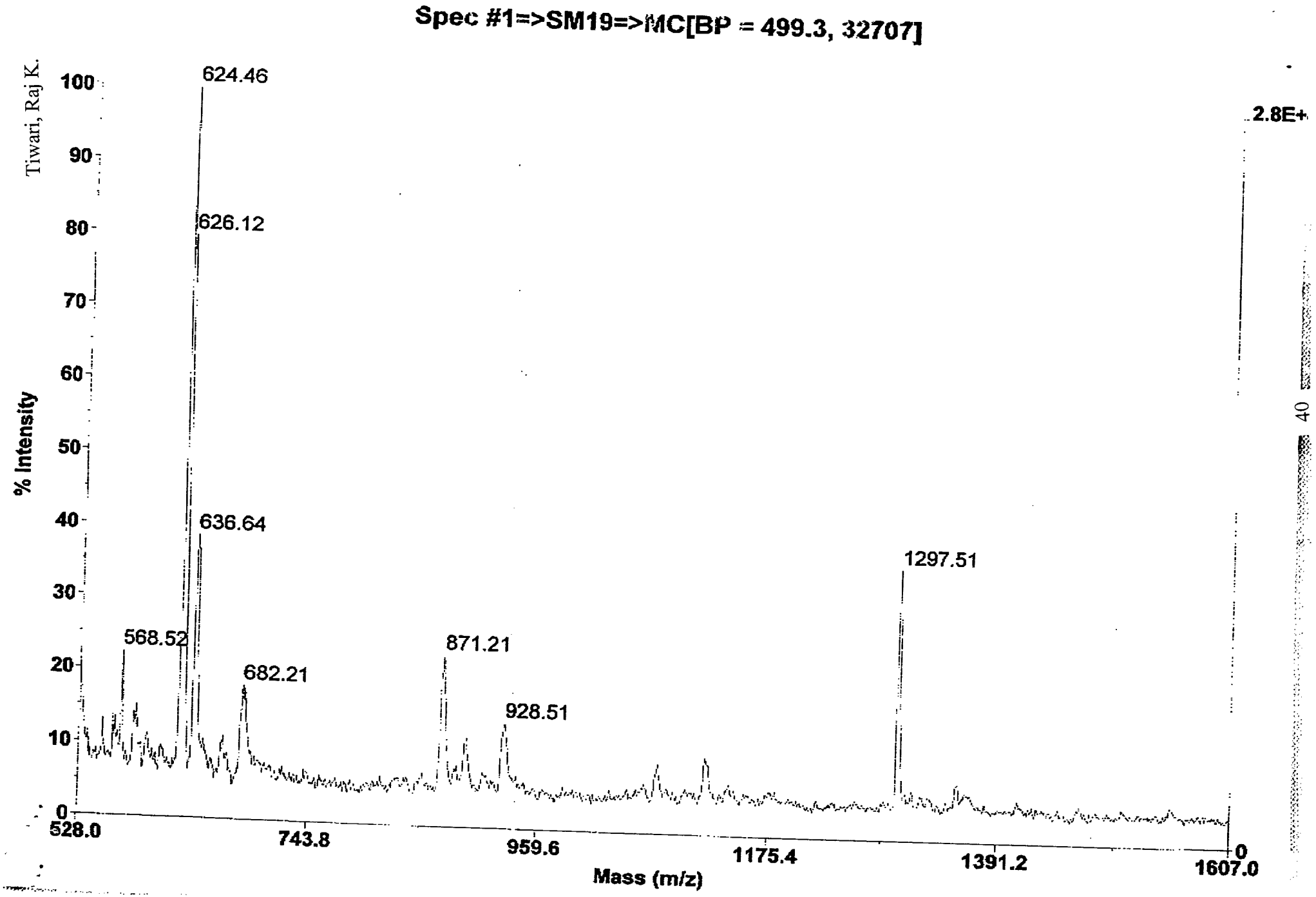
1289.99
661.15
668.39
883.44
879.37
845.73
1094.62
1305.38
1501.21
1712.19
801.28
1060.96
1134.89
% Intensity
Mass (m/z)
2.1E+
100
90
80
70
60
50
40
30
20
10
0
634.0
880.8
1127.6
1374.4
1621.2
1868.0
39

FIGURE 28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Tiwari, Raj K.
% Intensity
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80
70
60
50
40
30
20
10
0
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626.12
636.64
568.52
682.21
871.21
928.51
1297.51
528.0
743.8
959.6
1175.4
1391.2
1607.0
Mass (m/z)
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40
0

FIGURE 29

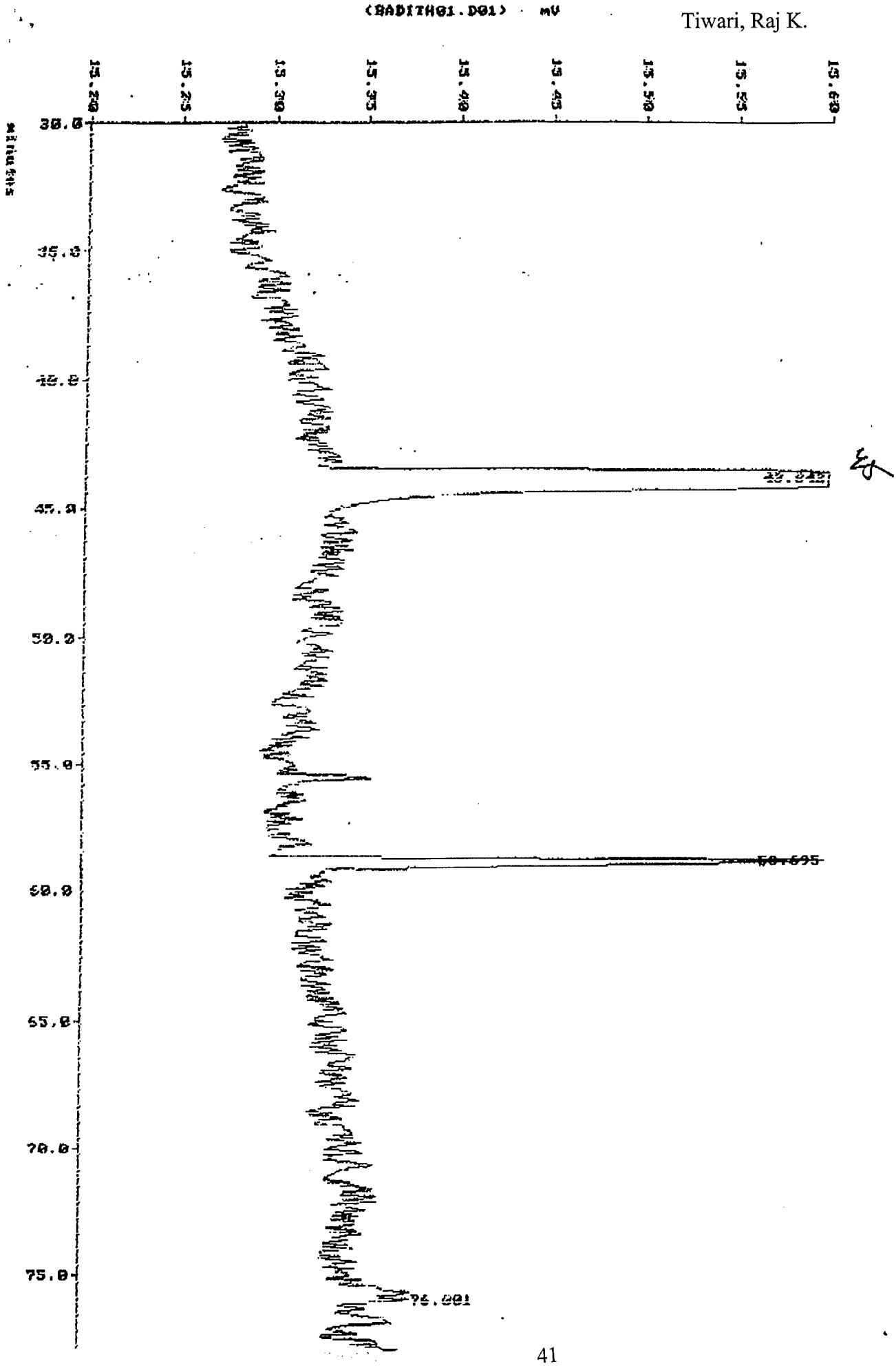

(BADITHG1.DG1) mV
15.20
15.25
15.30
15.35
15.40
15.45
15.50
15.55
15.60
Minutes
30.0
35.0
40.0
45.0
50.0
55.0
60.0
65.0
70.0
75.0
45.845
50.595
74.881

Fig. 30 Panning strategy for gp96-VSV-8 peptide complexes

VSV-8 peptide (RGYVYQGL) was loaded onto purified gp96 from MAT-LyLu tumor in a 50:1 molar ratio by incubating at 50°C for 10 min and RT for 30 min

↓

Library of single chain phage display combinatorial antibodies panned over gp96-VSV-8 peptide complexes

↓

Bound phages were eluted and passed over gp96-peptides complexes lacking VSV-8 peptide

↓

Unabsorbed phages were amplified and panned over gp96-VSV-8 peptide complexes again and repeated 3 times

↓

Absorbed phages at the last stage of panning eluted and tested for reactivity to gp96-VSV-8-peptide or gp96-peptide complexes or VSV-8 peptide alone by ELISA

Fig. 31. Reactivity of scFv differentially panned over gp96-VSV-8 complex and gp96

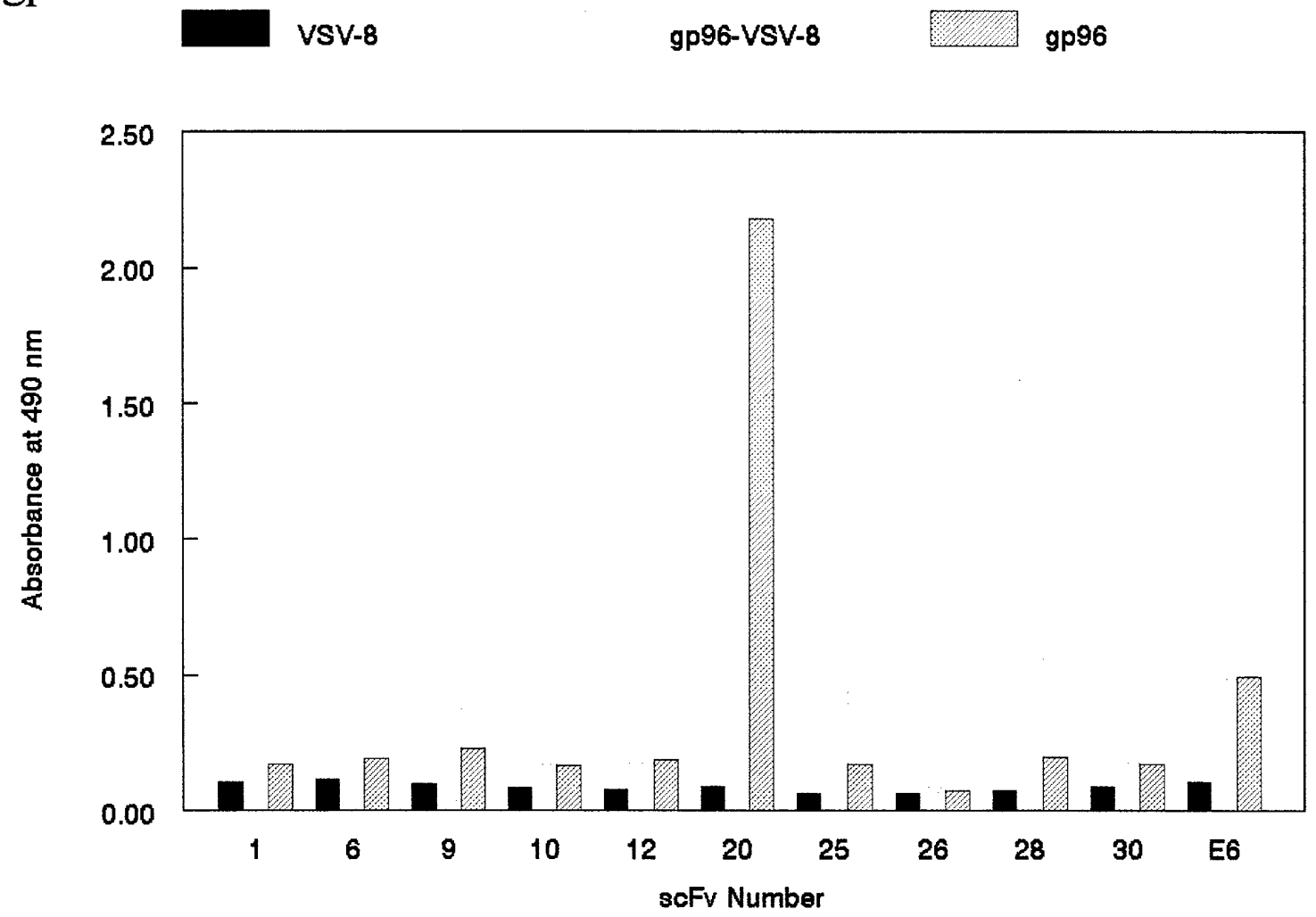


Fig. 32. Analysis of MAT-LyLu protein extracts using anti-gp96 antibody (+ control); E6 and F3 by western blots

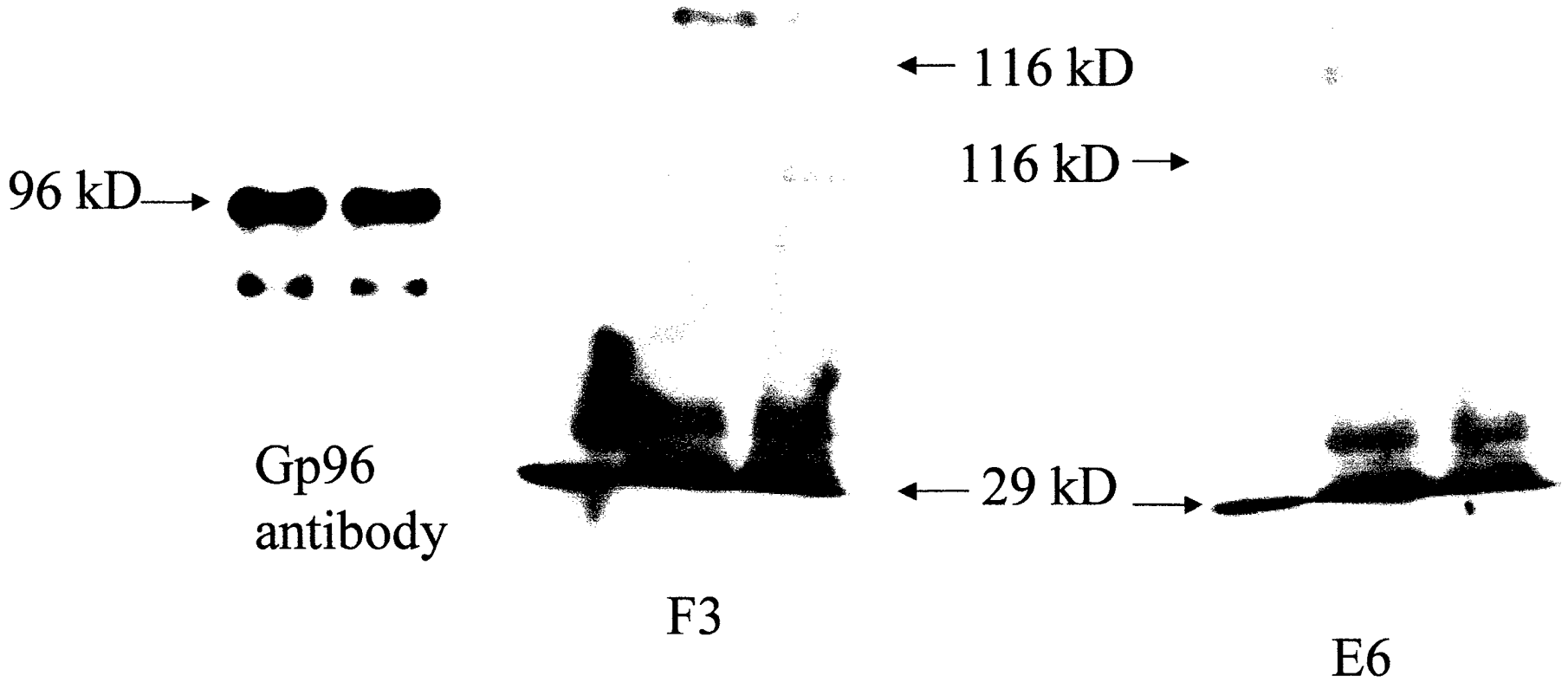
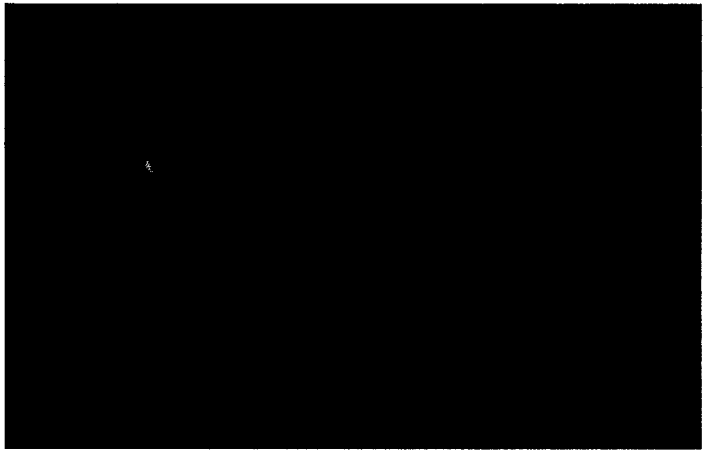
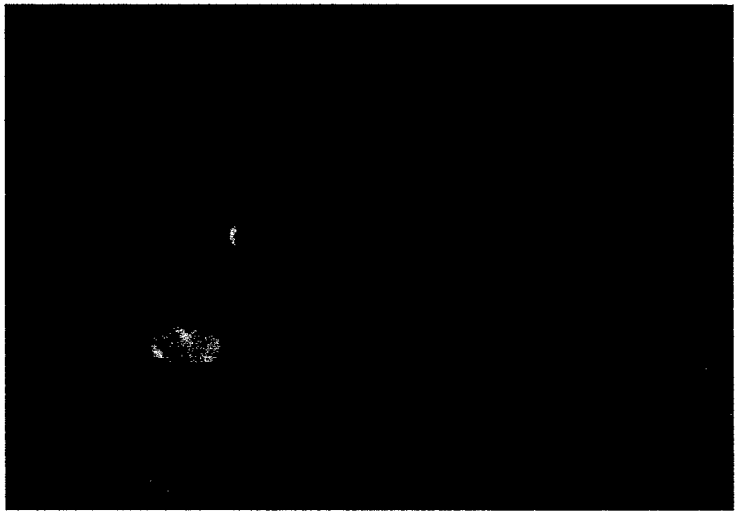


Fig.33. Analysis of binding of cell surface scFvs to MLL cells by immunofluorescence



MLL+BTA10

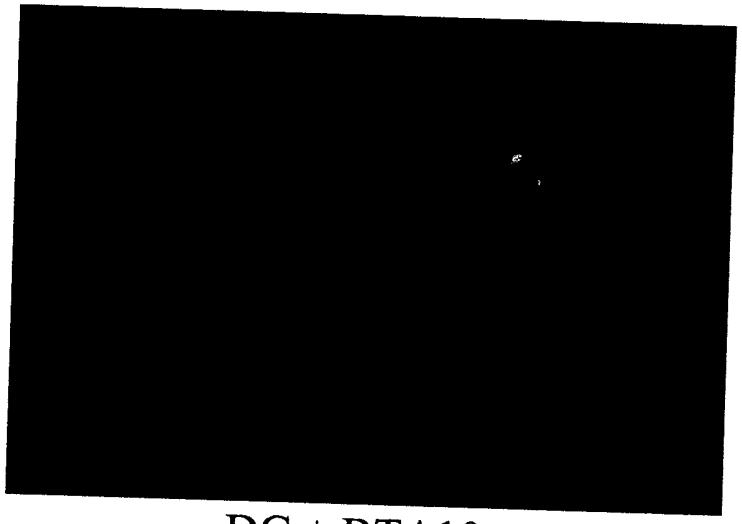
MLL+BTA20



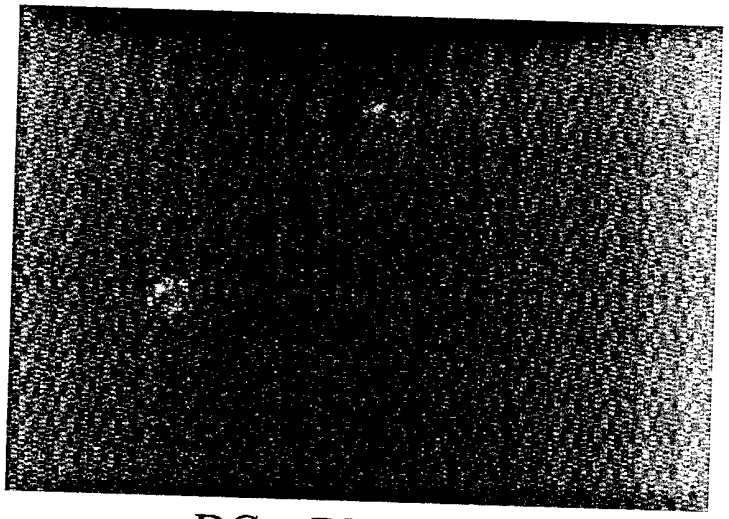
MLL+BTA35

MLL+BTA72

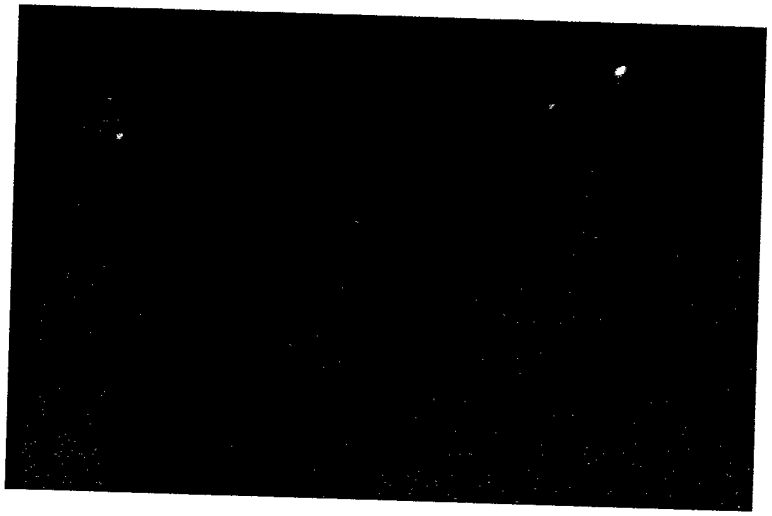
Fig.34. Analysis of binding of cell surface scFvs to DG cells by immunofluorescence



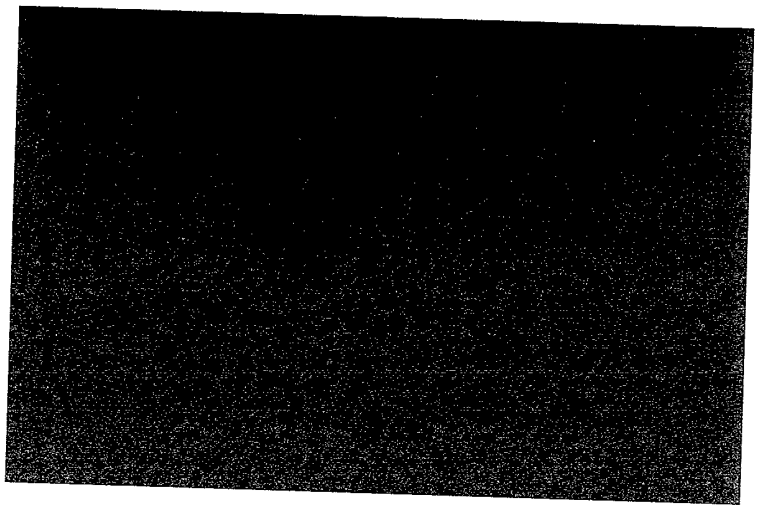
DG + BTA10



DG + BTA20



DG + BTA35



DG + BTA72

Table 1. V_H gene family, germline origin and V_H CDR3 amino acid sequence of the

Antigen	scFv#	V_H family	V_H-CDR3	Germline origin
MLL-derived	E6	V_H3	GKYIRSV	DP-38
gp96	F3	V_H3	GKYIRSV	DP-38
MLL cells	BTA20	V_H3	GMRPR	DP-38
	BTA35		LSSN	

Table 2. Amino acid sequence of synthetic peptides derived by panning with scFv E6

Library	Sequence
LX-8	YCQEGDSPRLCL
X-15	YQPPSDALRWILRLQ
	GQWQSGDRYWMETST

Table 3. Peptide sequence obtained from in vivo gp96 by MALDI analysis

#1: EVQLVESGGGLVQPG

#2: DIVLTQTPSSQAVSA

conspicuous infiltrate of CD4 and CD8 lymphocytes in the lung of mice treated with IL-13 + IFN-γ clones. The involvement of T lymphocytes was confirmed by the absence of therapeutic efficacy in athymic nude mice.

#1683 Combination effect of administration of NKT-cell stimulator, α-galactosylceramide and vaccination of IL-12 cDNA transduced tumor cell on subcutaneously inoculated tumor. Kogawa, K., Nishihori, Y., Tanaka, M., Hagiwara, S., Nakamura, K., Kuribayashi, K., Niitsu, Y. *4th Dept of Internal Medicine, Sapporo Medical Univ. Sapporo, 060-0061, Japan.*

α-galactosylceramide (KRN7000) has been reported to exhibit marked anti-metastatic activity through activation of Vα14NKT cells in mice model. However, its anti-tumor activity against subcutaneous tumor is subordinate to the one observed against metastasis. Therefore, we attempted to augment the anti-tumor activity of KRN7000 by combination with immune-gene therapy using IL-12 cDNA transduced tumor cells. Lewis Lung Carcinoma (3LL) cells were transfected with IL-12 bicistronic expression vector (TFG mIL-12, a kind gift from Dr. Tahara, H., Univ. of Pittsburgh) to obtain IL-12 producing 3LL(3LL-mIL12). 3LL cells (8×10^5) were first inoculated subcutaneously into the left back of BDF1 mice. Five days later, 2×10^7 of 3LL-mIL12 were inoculated into right back of the tumor-bearing BDF1 mice, KRN7000 100 μg/kg was intraperitoneally administered and the tumor size was measured at given time intervals. Results indicated that the combination of 3LL-mIL 12 inoculation and KRN7000 administration exhibits enhanced anti-tumor activity against 3LL subcutaneous tumor as compared to either one of 3LL-mIL12 inoculation or KRN7000 administration. Since we found increased expression of MHC class I, II and B7 on 3LL-mIL12 cells, enhancement of anti-tumor effects was considered to be ascribed to the modification of vaccinated tumor cells in addition to the production of IL-12 by tumor cells. These results suggest that combination of KRN7000 and IL-12 immuno-gene therapy is a potent anti-tumor therapy.

#1684 Enhancement of antitumor effect by additional transduction of IL-2 gene into IL-12-gene-transdused Lewis lung carcinoma. Tanaka M., Saijo Y., Tazawa R., Satoh K., and Nukiwa T. *Institute of Development, Aging, and Cancer, Tohoku University, Sendai, Japan.*

IL-2 and IL-12 are key cytokines that exert diverse immunological effects including strong antitumor activity. LLC/IL-12 (9.6 ng IL-12/10^6/24 hr), a mouse Lewis lung carcinoma cell line transduced murine IL-12 gene by retroviral vector, was subcutaneously transplanted into syngeneic C57BL/6 mice. Although reduction of tumor growth was observed by 65% compared to control cells, tumors did develop in all transplanted mice, showing only limited effect of IL-12 on LLC. In order to overcome the limited effect of IL-12 gene transduction, we investigated whether additional IL-2 gene transduction into LLC cells could enhance antitumor responses in mice. IL-2 gene was delivered to LLC/IL-12 using adenoviral vector, Adex-mIL-2 (74.2 ng IL-2/10^6/24 hr/100 M.O.I.). IL-2 and IL-12 co-transduced LLC developed tumors in only 3/12 mice, and reduced tumor growth by 90% compared to LLC/IL-12 or by 83% compared to IL-2 gene transduced LLC (LLC/AdexIL-2). To elucidate the mechanisms of this enhanced antitumor responses, histological and immunological analysis were performed. Splenocytes in mice transplanted either LLC/AdexIL-2 or LLC/IL-12/AdexIL-2 secreted higher IFN-γ compared to LLC or LLC/IL-12. Tumor-specific CTLs were induced only in mice transplanted LLC/IL-12/AdexIL-2. Immunohistrogical staining of tumor sections showed many infiltrations of CD4+ and CD8+ lymphocytes in LLC/IL-12/AdexIL-2 tumor. These findings suggest that local synergistic effect of IL-2 and IL-12 production resulted in the rejection of low immunogenic LLC. Combined transduction of IL-2 and IL-12 gene may provide a potential modality for immunogene therapy against low immunogenic cancers.

#1685 Synergy of SEB superantigen, MHC class II, and CD80 genes in immunotherapy of advanced spontaneous metastatic breast cancer. Pulaski, B.A., Terman, D., Muller, E., & Ostrand-Rosenberg, S. *Dept. of Biological Sciences, University of Maryland, Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250.*

No significant improvements in the treatment of metastatic breast cancer have been developed in the last 20 years and the prognosis for women with this disease remains poor. Progress in understanding the immune response, however, has led to renewed enthusiasm for immune-based anti-cancer therapies. In previous reports, we demonstrated that tumor cell-based vaccines expressing MHC class II and B7.1 (CD80) molecules reduced experimental (i.v.-induced) and established spontaneous metastatic disease, by activating tumor-specific $CD4^+$ T-lymphocytes. We now demonstrate, using the 4T1 mammary carcinoma, that a vaccine combining MHC class II and B7.1 molecules with SEB superantigen in two distinct immunotherapeutic regimens produces an even greater reduction in spontaneous metastatic disease and significant extension of mean survival time. The therapeutic effect is particularly noteworthy because: 1) spontaneous metastatic cancer by 4T1 progresses similarly in comparison to human metastatic mammary cancer, 2) our post-operative model demonstrates that early metastatic lesions are primarily responsible for morbidity, and 3) the disseminated metastatic disease is quite extensive prior to the initiation of immunotherapy in both regimens.

#1686 Irradiation enhances immunogenicity of cells expressing a tumor-specific T-cell epitope. Ciernik I.F., Romero P., Berzofsky J.A., and Carbone D.P. *Centre pluridisciplinaire d' Oncologie, the Ludwig Institute for Cancer Research, CHUV, Lausanne, Switzerland, National Cancer Institute, NIH, Bethesda, MD, and the Vanderbilt Cancer Center, Nashville, TN.*

p53 point mutations may represent potential tumor-specific cytolytic T lymphocyte (CTL) epitopes. We investigated a mutant tumor-specific p53-derived epitope overexpressed in cellular vectors with respect to its ability to induce mutant-specific CTL. P815 mastocytomas expressing the mutant p53 induced mutation-specific CTL in BALB/c mice after iv. injection. Syngeneic fibroblasts or fibrosarcomas endogenously expressing the mutant p53 were able to induce significant mutation-specific CTL only if they were irradiated prior to injection into BALB/c mice. Low dose gamma irradiation of fibroblasts did not alter the expression of cell surface molecules involved in immune induction, nor did it alter the short term viability of the fibroblasts. Radioactively labeled fibroblasts injected into mice after irradiation showed altered trafficking, suggesting that the *in vivo* fate of these cells may play a crucial role in their immunogenicity. These findings indicate that gamma irradiation can alter the immunogenicity of syngeneic normal as well as transformed fibroblasts *in vivo*.

#1687 Treatment of breast cancer with fibroblasts transfected with DNA from breast cancer cells. EP Cohen*, EF de Zoeten*, V Carr-Brendel*, D Markovic* and J Taylor-Papadimitriou#. **Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, Il 60612, USA and #Guy's Hospital London SE1 9RT England.*

A vaccine that prolonged the survival of mice with breast cancer was prepared by transfection of mouse fibroblasts with DNA from breast cancer cells. The underlying rationale was that the immunogenic properties of breast cancer associated antigens (TAAs), the products of mutant or dysregulated genes, would be enhanced if they were expressed by highly immunogenic cells. (Classic studies indicate that transfection of DNA from one cell type into another stably alters both the genotype and the phenotype of cells that take-up the exogenous DNA.) To investigate this question, we transfected LM mouse fibroblasts ($H\text{-}2^k$) modified to secrete IL-2 and to express $H\text{-}2K^b$-determinants with genomic DNA from a breast adenocarcinoma that arose spontaneously in a C3H/He mouse ($H\text{-}2^k$). The cells were then tested in C3H/He mouse for their immunotherapeutic properties.

The results indicated that tumor-bearing mice immunized with the syngeneic / allogeneic transfected cells survived significantly longer than mice in various control groups including mice treated with fibroblasts transfected with DNA from an unrelated tumor (melanoma). Similar beneficial effects were seen in C57BL/6 mice injected with a syngeneic breast carcinoma cell line (E0771) and fibroblasts transfected with DNA from E0771 cells. Supported by DAMD 17-96-1-6178.

#1688 Identification and characterization of mouse prostatic acid phosphatase: Implications for immunotherapy of prostate cancer in animal models. Dirk G. Brockstedt, Michael H. Shapero, Lawrence Fong, Edgar G. Engleman and Reiner Laus. *Department of Pathology, Stanford University School of Medicine, Stanford, CA, USA; Dendreon Corporation, Mountain View, CA, USA.*

In humans, prostatic acid phosphatase (PAP) is selectively expressed in prostatic tissue of normal and malignant origin. Thus far, a murine homologue for PAP has not been described. In order to evaluate its potential utility as a target antigen in animal models for PAP-directed immunotherapy of prostate cancer, we isolated the murine homologue of PAP (mPAP). The full-length cDNA clone encompasses a total of 1455 nucleotides containing the ORF for PAP, which encodes a 353-residue protein with a calculated molecular mass of 41 kDa. A comparison of protein sequences across species reveals 87% sequence identity with rat PAP and 80% sequence identity with human PAP. RT-PCR and Northern Blot analysis of mouse tissues reveals that mPAP expression, unlike its human homologue, is not restricted to the prostate, but is expressed in a wide variety of different tissues including thymus. Interestingly, additional studies in rats reveal that rat PAP exhibits an expression pattern that resembles the human but not mouse tissue distribution. To study the immunological consequences of this differential expression pattern in closely related species we produced recombinant rat and mouse PAP in a baculovirus expression system. We then immunized rats and mice with recombinant protein plus adjuvant and evaluated the induction of antigen-specific immunity. Immunization of mice with mPAP leads only to a weak antibody response in 20% of the mice. In contrast, immunization of rats with rat PAP leads to a significant antibody response in all rats. These findings suggest that the wide tissue expression of PAP in mice may lead to antigen specific tolerance. Thus, rat models are more suitable for evaluation of PAP-specific, prostate-directed immunity in animal models for prostate cancer.

#1689 The ER-resident heat shock protein gp96 is associated with HLA class I. Chen, Y.G., Mukhopadhyay, S., Yedavelli, S.P.K., Chatterjee-Kishore, M., Kishore, R. and Tiwari, R.K. *Department of Microbiology and Immunology, New York Medical College, Valhalla, NY 10595.*

The human leukocyte antigen (HLA) class I serves a key role in the presentation of endogenous peptides to cytotoxic T cells for generation of an immune response. The molecular steps involved in the selection and loading of the peptides on the HLA complex is still unclear. The present study was undertaken to specifically examine the association of the ER-resident, peptide chaperone, gp96, with HLA class I heavy chain in several lymphoma cell lines, including the T2 cell

line that lacks TAP expression. We detected this association using double-determinant immunoadsorbent assay (DDIA), co-immunoprecipitations and western blotting, and cell labeling studies. Protein complexes immunoprecipitated with anti-gp96 antibodies were shown to contain HLA class I heavy chain as detected by western blotting with biotinylated anti-HLA class I monoclonal antibody. These observations were validated using DDIA where the gp96-HLA complex in cell lysates was either captured by anti-gp96 or anti-HLA (w6/32) antibodies and traced with anti-HLA or anti-gp96 antibody. The human erythroleukemic cell line, K562 which lacks the expression of HLA Class I was used for comparison. Metabolic labeling of cells and immunoprecipitation of the labeled lysates provided further evidence of the association of gp96 with HLA class I heavy chain. This association may have implication in the selection of peptides to be loaded on to HLA complex. [Funded by US ARMY grant # 17-98-1-8534].

#1690 DNA as vaccine or therapeutic against cancer and viral infections. Moelling, K., Pavlovic, J., Schultz, J., Schuh, Th., Obraschall, E. and Heinzerling, L. *Institute of Medical Virology, University of Zurich, CH-8028 Zurich, Switzerland.*

Plasmid DNA encoding viral or tumor-associated antigens can induce protective immune response against challenge with virus or tumor cells when the DNA is injected intramuscularly (1-3). We are developing a DNA vaccine against malignant melanoma with a tumor and a metastasis model in mice using a melanoma-associated antigen and a number of cytokine-encoding DNA plasmids such as IL-2, GM-CSF, IL-12, and the costimulator B7.1. Depending on the type of cytokine, some tumor reduction was observed while metastasis in the lung was strongly inhibited. Systemic expression of cytokines was monitored over time and was long-lasting. Non-specific CpG's showed only transient effects. The protective immune response is analyzed using various genetically altered mice such as perforin-, Interferon Receptor (IFN-Rα)-, $CD8^+$-cells- and B-cells- knock-out mice. The mice were also immunized with viral DNA coding for Influenza A hemagglutinin and nucleoprotein, HIV-1 gag-pol, NC, bunya viral antigens and CMV antigens. Combination of viral antigen-encoding DNA with DNA encoding cytokines or peptides are under investigation. (1) Moelling, K., *J. Mol. Med.*, **75**, 242-246 (1997). (2) Moelling, K., *Cytokines Cellular and Molecular Therapy* **3**, 127-136 (1997). (3) Moelling, K., *Gene Therapy 5, 573-574 (1998).*

#1691 Development of a bladder cancer vaccine based on M344, a mucin-associated carbohydrate antigen. Bergeron, A., Champetier, S., LaRue, H. and Fradet, Y. *Cancer Research Center, Centre Hospitalier Universitaire de Québec, Pav. L'Hôtel-Dieu de Québec, Québec, (P.Q.), CANADA, G1R 2J6.*

Bladder cancer offers a unique opportunity to investigate cancer vaccines. Up to 75% of primary bladder tumors are superficial and, although treated effectively by transurethral surgery, recurrences occur in 60% of patients. Moreover, these tumors respond well to non-specific immunotherapy using BCG. We believe that vaccines based on bladder tumor-associated antigens could be used to prevent recurrence of this disease. M344 mAb reacts with an antigen that is expressed on more than 70% of superficial bladder tumors and is also found in premalignant lesions. We previously showed that M344 was a carbohydrate epitope found on a typical mucin. The first objective of this study was to further define the M344 epitope. In competition assays, we showed that M344 totally competed 49H8, a mAb directed against the Thomsen-Friedenreich (T-F) antigen. However, both mAbs react differently with other glycoproteins bearing the T-F antigen thus suggesting that M344 reacts with Galß1-3GalNAc presented in a specific environment. Carbohydrates, as T-cell independent antigens, often elicit incomplete immune response and thus can be advantageously replaced by antigen mimics such as surrogate peptides or anti-idiotype antibodies. A second objective of this study was to produce M344 mimicry antigens and analyze them in order to determine their ability to reproduce the antigenicity of the M344 natural epitope. The potential of these antigen mimics to confer tumor protection will need further study.

#1692 Interleukin 12 and B7.1 costimulatory molecules coexpressed from an adenoviral vector act synergistically to induce antitumor response and suppress tumor formation in Lewis lung carcinoma model. Z.S. Guo, L. Lee, A. Chen, F.L. Graham, and D.S. Schrump. *Surgery Branch, NCI, NIH, Bethesda, MD; and Depts of Biology and Pathology, McMaster University, Hamilton, Ontario, Canada.*

Effective alteration of the host-tumor relationship necessiates presentation of tumor antigen in the context of co-stimulatory molecules and cytokines. The Lewis lung carcinoma is a spontaneous, poorly immunogenic cancer which is highly malignant in syngeneic as well as allogeneic mice. These properties make it an ideal model to study lung cancer immunotherapy utilizing gene transfer techniques. Recently we evaluated the therapeutic efficacy of adenoviral vectors expressing both the IL-12 and B7.1 molecules, or either molecule alone in the Lewis lung carcinoma model in syngeneic C57BL/6 mice. Expression of functional IL-12, B7.1, or both molecules could be detected following transduction of murine and human lung cancer cells. Tumorigenicity was significantly reduced in Lewis lung carcinoma cells transduced with vectors expressing both IL-12 and B7.1 molecules, and to a lesser extent in cells transduced with either molecule alone. Mice with tumors derived from the double transduced LLC1 cells survived much longer than those with tumors derived from cells transduced with a blank vector. Experiments are in progress to evaluate resistance to challenge with parental tumor cells following inoculation of cells transduced with IL-12 and B7.1. These data suggest that combined costimulatory molecules and cytokines expressed from tumor cells may be efficacious for lung cancer immunotherapy.

#1693 Helper virus-free packaged herpes simplex virus type 1 amplicon vectors for GM-CSF-enhanced vaccination therapy of glioma. Herrlinger, U., Jacobs, A.H., Woiciechowski, C., Sena Esteves, M., Fraefel, C., Rainov, N.R., Breakefield, X.O. *Molecular Neurogenetics Unit, Massachusetts General Hospital, Boston, MA 02129; Department of Neurology, University of Tuebingen, Germany.*

Subcutaneous vaccination with glioma cells which are retrovirally transduced to secrete granulocyte-macrophage colony stimulating factor (GM-CSF) has proven effective in the mouse GL261 glioma model. As retroviral vectors only transduce dividing cells, clinical *ex vivo* gene therapy of gliomas with a low growth fraction seems to be difficult. To overcome this obstacle, a herpes simplex virus type 1 (HSV-1) amplicon vector, pHSVGM, expressing the GM-CSF gene from the HSV-1 IE4/5 promoter was constructed. This vector transduces both dividing and non-dividing cells. For mock infection, an amplicon vector encoding the *E. coli* lacZ gene was used. Both HSV-1 amplicon vectors were packaged helper virus-free. Infection of irradiated (35 Gy) GL261 cells with pHSVGM amplicon stocks did not cause any toxicity. The GM-CSF secretion during the first 24 h after infection was 34 ng/10^6 cells/24 h whereas mock-infected cells did not secrete any GM-CSF. In *in vivo* experiments with subcutaneous vaccination of C57BL/6 mice using 5×10^5 irradiated cells seven days prior to intracerebral implantation of 10^6 wildtype GL261 cells, 60% of the animals vaccinated with pHSVGM-infected GL261 cells were long-term survivors. However, in the groups vaccinated with retrovirally transduced GL261 cells or mock-infected GL261 cells 16–20% of the animals were long-term survivors. Vaccination with wildtype GL261 cells did not induce long-term survival. In conclusion, helper virus-free packaged HSV-1 amplicons vectors appear to be promising tools for cytokine-enhanced vaccination therapy of glioma.

#1694 Primary T-cell and activated macrophage response associated with tumor protection using peptide/poly-N-acetyl glucosamine (p-GlcNAc) vaccination. Maitre N, Stack A, Brown JM, Demcheva M, Kelley JR, Vournakis J, Cole DJ. *MUSC Dept of Surgery and CMSB, Hollings Cancer Center, Charleston SC.*

Effective anti-tumor vaccination may depend on the ability to generate an early cell-mediated response. We have previously shown that vaccination with the F2 gel formulation of p-GlcNAc results in cell-mediated tumor protection. The purpose of this study was to evaluate the mechanisms of F2 gel/peptide generation of this response using a murine EG.7-OVA tumor model. C57BL/6 mice were vaccinated with 200 μl in the base of tail/footpad using either F2 gel alone, 200 μg of SIINFEKL minimal peptide (OVA) in F2 gel, or OVA in PBS. Splenocytes at 24 and 48 hours post-vaccination were assayed for cell surface and intracellular markers, and day 10 were tested for a primary CTL response using the LDH cytotoxicity assay. Macrophages were then depleted prior to vaccination (mannan, 10 mg/ml i.p). Vaccination with F2 gel/peptide resulted in a primary T-cell response (25% tumor specific lysis). By 48 hrs, splenic T-cells had increased 4-fold as compared to B cells. Presence of an increased Th1 CD4 helper population was demonstrated by Interferon-γ production. CD4 cells were activated at 24 and 48 hours as shown by IL-2 receptor α chain expression (from 2% to 15% at 48 hrs). Activated splenic macrophages increased from 3 to 8% by 10 hrs with B7-2 expression doubled. Pre-vaccination macrophage depletion abolished any tumor-specific primary CTL response. F2 gel/minimal peptide tumor vaccination primed the immune system in an antigen specific manner with a primary T cell response involving macrophage presence and activation as well as induction of Th1 CD4 cells.

#1695 Induction of specific T cells tolerance to MUC1 antigen in MUC1 transgenic mice. Chen, DS., Koido, S., Li, YQ., Rowse, GJ.,* Gendler, SJ.,* and Gong, JL. *Cancer Pharmacology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115. *Samuel C. Johnson, Medical Research Building, Mayo Clinic Scottsdale, Scottsdale, AZ 85259.*

The C57BL/6 mice transgenic for human MUC1 (MUC1 Tg) have been developed to investigate anti-MUC1 tumor immunity in an animal that expresses MUC1 as a self -antigen. Previous studies showed that MUC1 transgenic mice were tolerant to self-MUC1 tumor associated antigens. In this study, we have investigated the mechanisms of tolerance to human DF3/MUC1 tumor-associated antigen in MUC1 transgenic mice. Immunization with different doses of purified MUC1 antigen or irradiated MUC1-positive tumor cells (MC38/MUC1) was unsuccessful in inducing anti-MUC1 immunity. The MUC1 Tg mice were immunized with MUC1 antigen and MC38/MUC1 shown CD8 T cells at different level reduction, and lack of T cell co-stimulation. By contrast, there were no significant difference in anti-MUC1 antibody secreted in wild-type and MUC1 Tg mice after MC38/MUC1 immunization. Furthermore, compared with irradiated MC38/MUC1 and MC38/β gal immunization, MUC1 Tg induces tolerance to specific MUC1 antigen, not β gal. These findings represent the tolerance induction in adult MUC1 Tg mice, and indicated that specific T cell suppressers play an important role in immune tolerance to MUC1 tumor-associated antigen.

IMMUNOLOGY/PRECLINICAL AND CLINICAL 8: Tumor Antigens II

#3102 Accuracy of TA90 immune complex in the detection of breast carcinoma. Habal, N., Gupta, R., Yee, R., Stern, S., Brennan, M., Brenner, R., Hansen, N., Giuliano, A., Morton, D. *John Wayne Cancer Institute, Santa Monica, CA 90404.*

We have identified a 90-kD glycoprotein serum tumor marker present as a circulating immune complex (TA90-IC) in patients with a variety of solid neoplasms, including breast cancer. This study evaluated the efficacy of TA90-IC in detecting breast cancer, in comparison to the gold standard of mammography. One hundred and forty-seven women (ages 26–82 years) were referred for open breast biopsy because of an abnormal mammogram (n=100) or palpable breast mass (n=47). Fifty-four (37%) patients were younger than 50 years. Preoperative serum samples were tested blindly for TA90-IC using an enzyme-linked immunosorbent assay (ELISA). A positive result was an optical density > 0.410 at an absorbance of 405 nm. Mammogram reports were obtained from the patient's chart and classified as suspicious (indeterminate readings included) or benign. Histopathologic results were classified as positive (infiltrating and intraductal cancer) or negative. Sixty-six (45%) patients had a breast cancer. When compared with mammography, TA90-IC was less sensitive (62% vs. 83%) but more specific (90% vs. 44%) and had a lower false positive rate (10% vs 56%). The difference between TA90-IC values and mammography results was significant ($p<0.0001$ based on McNemar's test.) A combination of tests improved the accuracy of screening (71% for TA90-IC plus mammography vs. 62% for mammography alone) and lowered the rate of false-positives (46% vs. 56%). We conclude that combining TA90-IC and mammography may help reduce the number of unnecessary tests and breast biopsies. The role of TA90 in screening for breast cancer and monitoring progression of disease will be evaluated in a multicenter trial.

#3103 Cell-surface expression of an immunogenic ganglioside (GM2) in pancreatic adenocarcinoma. Chu, K.U., Ravindranath, M.H., Nishmoto, K., Bilchik, A., and Morton, D.L. *John Wayne Cancer Institute, Santa Monica, CA 90404.*

Effective antitumor immunotherapy requires well-exposed immunogenic targets on the surface of tumor cells. Human pancreatic adenocarcinoma cells express gangliosides on their surface. By analyzing antibody levels against different gangliosides, we recently demonstrated that ganglioside GM2 is immunogenic in patients with pancreatic ductal adenocarcinoma. However, these tumor cells have a complex glycocalyx surface component that may mask the expression of GM2. The present study was undertaken to determine whether GM2 antigens are expressed on commonly studied pancreatic adenocarcinoma cell lines and whether the cell-surface expression of GM2 is altered by enzymatic cleavage of the glycocalyx. An anti-GM2 monoclonal antibody in a cell-suspension ELISA was used to examine the expression of GM2 in four pancreatic adenocarcinoma cell lines obtained from ATCC (SU.86.86, CFPAC-1, ASPC-1, and BXPC-3). Cell lines were analyzed before and after enzymatic cleavage of the glycocalyx using O-glycosidase with or without pronase. All four cell lines expressed GM2 in varying amounts that increased when cells were treated with enzymes. Addition of pronase after O-glycosidase did not affect the expression of GM2. These data suggest that GM2 is expressed in pancreatic adenocarcinoma cell lines but is masked by the complex glycocalyx on the cell surface. For effective immunotherapy in patients with pancreatic adenocarcinoma, better exposure of these potential immunogenic antigens on the surface of tumor cells may be needed, and this may be possible by combination chemotherapy that downregulates the expression of the complex glycocalyx.

#3104 Unusual and novel features of peptide binding to MHC Class I molecules. Apostolopoulos, V., McKenzie, I.F.C., Teyton, L., Chelvanayagam, G., and Wilson, I.A. *The Austin Research Institute, Heidelberg, VIC 3084, Australia; The John Curtin School of Medical Research, Canberra, ACT 2601, Australia; The Scripps Research Institute, Department of Molecular Biology, La Jolla, CA 92109.*

MUC1 is a high molecular weight glycoprotein overexpressed by adenocarcinomas. We have been using mannan-MUC1 for the selective delivery of MUC1 peptides to the Class I pathway. MUC1 peptides are presented by a variety of Class I molecules and can generate CTLs. We have described the 9mer epitopes presented by different H-2 Class I molecules and by HLA-A2. For the most part, the 9mer peptides have unusual features in that they lack defined anchors and bind with low affinity. Nonetheless, high avidity CTLs are produced. We also noted that MUC1 9mer peptides were not essential as 5- 8mer peptides could also bind. In addition to VNTR peptides, several mutations and mimics have been generated. This approach leads to the generation of more powerful anti-MUC1 CTLs. The MUC1 peptides have other novel features. They bind in an unusual fashion, in that the mid and C-terminal regions loop out of the groove more than other peptides and are accessible to MUC1 antibody while the N-terminus is buried. Indeed, MUC1 peptides are the only peptides which are accessible to antibody while in the groove of Class I molecules, although other antibodies can react with peptide/MHC molecules or with a MHC dependent configuration. The results clearly indicate that MUC1 is binding in an unusual manner; structural studies in progress confirm this. The studies demonstrate that peptides do not have to be deeply bound within the groove of Class I molecules, indeed low affinity binding peptides can generate CTLs. Thus, a different mode of peptide binding occurs which may have implications in immunity, and in tolerance induction.

#3105 Identification of tumor associated antigens using purified gp96-peptide complexes and the synthetic combinatorial single chain (scFv) phage display antibody library. Mukhopadhyay, S., Yedavelli, S.P.K., Noronha, J., Ferrone, S., Tiwari, R.K. *Department of Microbiology and Immunology, New York Medical College, Valhalla, NY, 10595.*

Tumor derived purified preparation of heat shock protein, gp96, in contrast to non tumor derived gp96 have tumor protective effects. In the absence of any structural difference between gp96 from tumor and non-tumor tissues and the observation that gp96 is associated with a large number of cellular peptides and preparations of gp96 stripped of their associated peptides lose their immunogenicity, it was hypothesized that identification and isolation of peptides associated with gp96 would lead to a standardized treatment. Towards this end, we describe the use of a synthetic combinatorial phage display antibody library in identifying tumor specific gp96 associated peptides. The initial library used for differential panning over purified gp96-peptide complexes from prostate tumor and normal liver contained over 10^8 distinct single chain antibody fragments. After four rounds of differential panning, three hundred phages that specifically adhered to tumor derived gp96-peptide complexes were eluted and individually analyzed. Five phages that specifically reacted to tumor derived gp96-peptide and not to gp96-peptide complexes derived from normal prostate, normal liver were identified. These single chain antibodies provide a useful probe to isolate tumor associated peptides that mitigate tumor rejection. (Funded by the US ARMY Grant # 17-98-1-8534)

#3106 Destruction of human malignant brain tumors by irradiated TALL-104 cells and their movement through normal rat brain: A cellular therapy paradigm. Kruse, C.A., Lamb, C., Gup, C., Hogan, S., Gomez, G., Kleinschmidt-DeMasters, B.K., Visonneau, S. and Santoli, D. *Univ of Colo Health Sci Ctr, Denver, CO 80262 and The Wistar Institute, Philadelphia, PA 19104.*

We are conducting preclinical studies with a human non MHC-restricted killer T cell line, TALL-104, in anticipation of its use in cellular immunotherapy trials for primary malignant brain tumors. The irradiated TALL-104 leukemic cell line was shown to lyse tumor cells across species barriers. In this study we have 1) quantitated in vitro brain tumor cell lysis and cytokine secretion upon TALL-104 cell:brain tumor cell co-incubation, and 2) estimated the damage to normal rat brain and trafficking patterns of the TALL-104 cells when placed intracranially. *In vitro* co-incubation of lethally-irradiated TALL-104 cells with brain tumor cell lines resulted in significant tumor cell lysis. Cytokines TNF-α, TNF-β, γ-IFN, or GM-CSF were variably secreted. Irradiated TALL-104 cells were placed into normal cannulated rat brain multiple times (10^6/injection, days 1, 3, and 7) to mimic the procedure we would follow in humans with a reservoir/catheter system. On days 8, 11 & 15 following the first infusion, histologic analyses of brains showed similar findings. The TALL-104 cells did not cause a widespread allergic encephalitic reaction. Neuronal damage was not evident. Focal sterile abscesses formed at the site of instillation. TALL-104 cells trafficked from the site of instillation through neuropil; a small percentage trafficked into contralateral brain. TALL-104 cell exit was at perivascular and leptomeningeal spaces. Immunostains of rat brain with anti-rat and anti-human CD3 differentiated human TALL-104 cells from endogenous immune rat T cells. Limited immune cell infiltrates (lymphocytes, plasma cells, and small numbers of eosinophils) were present, indicative of an endogenous immune reaction, however, it is unclear if this was a reaction to xenogenic cells, TALL-104 cellular debris, or to viable TALL-104 cell presence. At day 15, cellular debris was being cleared and the instillation cavity was collapsing. (Supported by NIH NS28905 & Univ of Colo Cancer Ctr to CAK & by DAMD17-97-C-7056 to DS).

#3107 The SART-1 antigens as an appropriate vaccine candidate for cancer patients. Sasatomi T., Shichijo S., Niiya F., Yamana H., Ogata Y., Shirouzu K. and Itoh K. *Kurume University School of Medicine, 67-Asahi-machi Kurume 830-0011, Fukuoka, JAPAN.*

We recently reported the SART-1 gene encoding both the SART-1_{259} antigen expressed in the cytosol of squamous cell carcinomas and adenocarcinomas, and the SART-1_{800} antigen expressed in the nucleus of the majority of proliferating cells. (Shichijo et al, J. Exp. Med., 187, 277–288, 1998) The SART-1_{259} antigen was recognized by the HLA-A24-restricted cytotoxic T lymphocytes (CTLs). This study investigated the expression of SART-1_{259} and SART-1_{800} antigens in cancer tissues. The SART-1_{259} antigen was detected in the cytosol fraction of cancer cell lines, and cancer tissues, but not in non-tumorous tissues. The SART-1_{800} antigen was expressed in the nuclear fraction of almost all the cancer cell lines, cancer tissues and few of non-tumorous tissues. The SART-1_{259}^{+} cancer cells were recognized by the HLA-A24 restricted and SART-1 specific CTLs. Therefore, the SART-1_{259} antigens could be an appropriate vaccine candidate for cancer patients.

difficult because these antigens are autoantigens. To get maximal benefit from the adjuvant component of cancer vaccines we have tested whether combinations of the optimal adjuvants induced an improved immune response compared to QS-21 alone. Twelve different adjuvant combinations were compared for their ability to augment 1) antibody responses against GD3 and MUC1 and 2) T cell responses against GD3, MUC1 and KLH. Five adjuvant combinations were superior to QS-21 for induction of IgM and IgG antibodies against MUC1 and GD3: QS-21 plus bacterial nucleotide CpG, QS-21 plus monophosphoryl lipid A (MPL), QS-21 plus non-ionic block copolymer CRL-1005, TiterMax plus CpG, and CpG plus MPL. Antibody responses were documented both by ELISA against purified antigens and by FACS for cell surface reactivity. There was no evidence for T cell immunity against GD3 or MUC1. The antibody responses were however strongly correlated with IFN-gamma release and to a lesser extent DTH and IL4 release against KLH. These results demonstrate that combinations of immunological adjuvants are able to augment antibody and T cell responses beyond that attainable with any single adjuvant. Supported by a grant from the Milstein Foundation.

#4436 ULTRASTRUCTURAL STUDY OF ANTIGEN PRESENTATION BY FUSION WITH DENDRITIC AND BREAST CANCER CELLS. Jianlin Gong, Y H Xiu, L Zhang, D S Chen, S Koido, Z K Wu, D Avigan, and D Kufe, *Dana-Farber Cancer Institute, Harvard Med Sch, Boston, MA*

Our previous studies have demonstrated that hybrid cells created by the fusion of dendritic cells (DC) with MC38 carcinoma cells expressing the MUC1 carcinoma-associated antigen are effective in the treatment of established MUC1-positive pulmonary metastases in mice. Significantly, immunization with fusion cells abrogates immune unresponsiveness against MUC1 in MUC1 transgenic mice. More recent studies have demonstrated in vitro activation of autologous T cells by human DC-breast cancer fusion cells. To investigate in part the base for induction of the anti-tumor immune response, we have characterized the ultrastructure of fusion cells by scanning electron microscopy (SEM). The surface of the fusion cell exhibits DC characteristics with veiled processes. Transmission electron microscopy (TEM) has further demonstrated that the fusion cells express the MHC class II compartments (MIIC) unique to antigen-presenting cells. Importantly, ultrathin cryosections of fusion cells subjected to dual immunogold labeling demonstrated that MHC II and MUC1 antigen are present in the endoplasmic reticulum, Golgi apparatus, multilaminar MIIC, plasma membrane and on the surface of the fusion cells. These findings indicate that MHC II and MUC1 are newly synthesized by the fusion cells. In this context, the loading of antigen is more efficient and the antigen can be presented more effectively.

#4437 ACTIVE IMMUNIZATION AGAINST THE ANGIOGENIC RECEPTOR FLK-1 INHIBITS TUMOR METASTASIS. Yiwen Li, M. Wang, K. King, A. Hooper, H. Sun, A. Santiago, J. Overholser, P. Bohlen, and D. J Hicklin, *ImClone Systems Inc, New York, NY*

Angiogenesis plays a key role in growth and metastasis of tumors. The vascular endothelial growth factor (VEGF) receptor Flk-1 is over-expressed on tumor-associated endothelial cells and is critical for the neovascularization of tumors. We hypothesize that an immune response can be elicited against Flk-1 expressed on tumor endothelium that will lead to inhibition of angiogenesis and tumor growth. To test this hypothesis, C57BL/6 mice were vaccinated with dendritic cells pulsed with Flk-1 protein (DC-Flk-1), a control protein, or with vehicle alone and then tested for antibody and T cell responses against Flk-1. High levels of anti-Flk-1 antibody and CTL activity were found in mice vaccinated with DC-Flk-1 but not in control mice, indicating that tolerance to self Flk-1 can be broken using this immunization approach. The CTL were found to recognize and lyse Flk-1 positive endothelial cell lines in vitro. We used the Lewis lung carcinoma metastasis model to determine whether active immunization against Flk-1 can inhibit angiogenesis and tumor growth. Mice immunized with DC-Flk-1 dramatically inhibited the growth of metastases in the lungs compared to control mice. About 60% of DC-Flk-1-immunized mice became tumor free while all mice in the control groups developed metastases in the lungs. Moreover, depletion of CD8 but not CD4 T cells abrogated the anti-tumor effect of DC-Flk-1 vaccination, suggesting that CD8 T cells were involved in the anti-Flk-1 response. The effect of active immunization against Flk-1 on normal angiogenesis processes was also investigated. No difference was found in wound healing in mice immunized with DC-Flk-1, while the reproductive function in immunized female mice was partially inhibited as tested in a pregnancy experiment. These data suggest that active immunization against angiogenesis-related targets may be a potentially useful approach to treatment of malignancies.

#4438 A NEW VARIANT OF CYSTEIN-RICH FGF RECEPTOR (CFR-1) SPECIFICALLY EXPRESSED ON TUMOR CELLS. Frank Hensel, Veit Krenn, Astrid Kloetzer, Angela Bachi, Matthias Mann, Hans Konrad Mueller-Hermelink, H. Peter Vollmers, *Institute of Pathology, Wurzburg, European Molecular Lab., Heidelberg, Germany*

Growth factor receptors are often overexpressed or modified on malignant cells. A new 130 kD variant of CFR-1 (cystein-rich fibroblast growth factor receptor) was detected by a human monoclonal autoantibody, isolated from a patient with a adenocarcinoma of the stomach. CFR-1 is highly homologous to MG-160 and ESL-1, which are ubiquitously expressed on human tissues. In contrast, the new CFR-1 variant is overexpressed on most tested human tumors but on normal tissue it can only be detected on stomach mucosa, adrenal cortex and in the Golgi-apparatus of collecting tubes of kidney. Binding of the monoclonal antibody 103/51 to CFR-1 induces elevated cell proliferation of various stomach cancer cell lines by tyrosin-phosphorylation of several proteins. The epitope was determined to be a N-linked carbohydrate side chain. The unique expression of this new molecule offers not only the possibility to study specific proliferation processes of malignant cells but is also of diagnostic and therapeutical value.

#4439 A MUTATION IN THE MALIC ENZYME GENE GENERATES AN ANTIGEN RECOGNIZED BY ANTI-TUMOR CTL OF A LUNG CARCINOMA PATIENT WITH LONG SURVIVAL. Vaios Karanikas, P Weynants, J-F Baurain, R Chiari, D Colau, J Thonnard, A Van Pel, T Boon, and P Coulie, *Ludwig Institute for Cancer Research, Brussels Branch, and Cellular Genetics Unit, Univ Catholique de Louvain, Brussels, Belgium*

The identification of tumor specific antigens recognized by cytotoxic T lymphocytes (CTL) on melanoma cells has introduced the possibility of identifying lung carcinoma specific antigens. The aim of this study was to identify tumor specific antigens recognized by CTL in the blood of a patient with lung cancer. A lung carcinoma cell line was derived from tumor material resected from a non-small cell lung cancinoma patient who was enjoying a favorable clinical course, being alive 10 years after partial resection of the primary tumor. Two HLA-A2 restricted CTL clones were derived from an autologous mixed lymphocyte-tumor culture and were used to screen a cDNA library prepared with mRNA from the autologous tumor. The antigen was identified as a mutated peptide encoded by the cytoplasmic form of malate dehydrogenase, which links the glycolytic and citric acid cycles. The CTL clones recognized the mutated peptide in an HLA-A2 restricted manner. Soluble HLA-A2 peptide- complexes were prepared and used to label blood lymphocytes of the patient. The frequency of the malic specific CTL was found to remain constant throughout the 10 years at about 0.4% of circulating CD8. The tetramer positive CTL were predominantly CD45-RO and HLA-DR positive and expressed killer inhibitory receptor molecules on their surface. These CTL were cloned and restimulated in vitro. Expansion was observed for all of them, indicating that they were not anergic. These results demonstrate that some cancer patients may develop a strong CTL response, measurable in the blood, against truly tumor specific antigens.

#4440 A NOVEL GENE ENCODING AN IMMUNOGENIC TUMOR ANTIGEN IDENTIFIED FROM PROSTATE CANCER CELL LINE USING SEREX. Yaling Zhou, P. A Lodge, S. J Monahan, S. Gil, M. Toth, A. L Boynton, and M. L Salgaller, *Northwest Biotherapeutics, Inc, Seattle, WA*

Serological Immunogenic tumor antigens by recombinant expression cloning (SEREX) plays a critical role in developing efficient therapeutic strategy for treatment of cancer. Lambda phage-based cDNA expression libraries were constructed from two prostate cancer cell lines, LNCaP and PC3. Using SEREX, we identified a total of 8 genes whose expression elicited antibody responses in prostate cancer patients. Two were derived from the LNCaP cDNA library, and the remaining 6 from our PC3 cDNA library. Of the 8 genes, 5 represented known genes in the GenBank database, 2 were previously uncharacterized genes, and I showed sequence homology to a mouse gene. The sequence feature and the expression of a novel gene, P9, was further investigated for this study. The P9 cDNA is 3,256 bp in length and contains a complete open reading frame of 2,751 bp encoding for a primary translation product of 917 amino acids. Northern blot hybridization detected a single species of approximate 3.3 kb P9 mRNA in normal prostate cells. However, the expression of the P9 is reduced in most prostate cancer cell lines, including LNCaP, PC3, and TSUPr-1. Similarly, via RT-PCR, we detected decreased P9 expression in most prostate tumor tissues examined as compared with normal prostatic specimens. The result suggests that P9 may function as a tumor suppressor for prostate cancer. Studies are underway to more comprehensively analyze this and other genes for their tissue and tumor distribution, their antibody frequency in patient sera, and their potential capacity to stimulate specific T cell immune responses.

#4441 IDENTIFICATION OF PROSTATE CANCER ASSOCIATED IMMUNOGENS USING SYNTHETIC COMBINATORIAL SINGLE CHAIN (SCFV) PHAGE DISPLAY ANTIBODY LIBRARY. Baditthe Ashok, Yuangen Chen, Sai Yedavelli, Jacintha Noronha, Abraham Mittelman, and Raj K Tiwari, *New York Med Coll, Valhalla, NY*

Immunotherapy in spontaneous prostate cancer is a major challenge. We used the R3327 experimental rat model to examine if immune response capable of causing tumor regression can be generated. Protective tumor immunity can be generated in these animals by tumor induction and by tumor derived purified preparations of heat shock protein gp96. Gp96 mediated protective immunity was specific and more pronounced with the metastatic subline, MAT-LyLu than the non-metastatic Dunning G cell line. Since gp96 is a peptide chaperone in the endoplasmic reticulum, we utilized the synthetic combinatorial phage display library to differentially select out peptides that specifically bound to tumor derived gp96. We successfully selected phages that reacted specifically to tumor derived gp96-peptide complexes and had minimal reaction to non-tumor derived gp96-peptide complexes. Using soluble antibodies derived from these reactive phages we were able to specifically immunoprecipitate a protein of molecular weight (~170 kDa) by metabolically labeling MAT-LyLu cells. This protein was not a cell

surface protein. Using the phage display combinatorial antibody library and panning over MAT-Ly Lu , we were able to identify distinct set of phages that reacted with cell-specific surface proteins but did not react with purified gp96-peptide complexes. We conclude that the single chain phage display antibody library is a useful reagent to identify intra-cellular and cancer associated cell surface markers and tumor rejection peptides associated with chaperone proteins, gp96. (Funded by the US ARMY Grant # 17-98-1-8534)

#4442 HUMAN ENDOGENOUS RETROVIRUS, HERV-K 10, IS EXPRESSED IN HUMAN BREAST CANCER. Feng Wang-Johanning, M B Khazeali, Theresa V Strong, William E Grizzle, and Albert F LoBuglio, *Univ of Alabama at Birmingham, Birmingham, AL*

As part of an ongoing search for novel tumor antigens, we have analyzed the expression of human endogenous retrovirus (HERV) genes in human cancer tissues. We examined mRNA expression of ERV3, HERV-E 4-1, and HERV-K 10 in breast tissues by reverse transcription-polymerase chain reaction (RT-PCR) and RNA *in situ* hybridization. HERV-K 10 was expressed in most human breast cancer cell lines and most frozen stored samples of breast adenocarcinoma, but not in normal human mammary epithelial cells and tissues. Expression of HERV-K 10 *env* mRNA was higher in breast tissue containing a higher proportion of tumor cells and in breast cancer cell lines stimulated with female hormones. The expression of HERV-K 10 *env* mRNA in breast adenocarcinoma was confirmed by RNA *in situ* hybridization using an HERV-K 10 *env* specific antisense probe. Several RT-PCR fragments of HERV-K 10 from breast cancer tissues were cloned and sequenced. The highest alignment scores for most sequenced clones were with the previously reported HERV-K 10 *env* gene sequence. Some cloned fragments, which were subcloned into a prokaryotic expression vector, produced stable full-length HERV-K 10 env proteins. We derived polyclonal antibody directed against HERV-K10 env protein, and used the antibody to demonstrate HERV-K 10 env protein expression in tumor epithelial cells. Thus, HERV-K-like transcripts capable of producing stable proteins are commonly expressed in breast cancer. The expression of HERV-K 10 *env* mRNA transcripts and protein in malignant but not normal breast tissues may provide a tumor-associated antigen for diagnostic and therapeutic applications.

#4443 IMMUNE RESPONSES OF BREAST CANCER PATIENTS TO MUTATED EPIDERMAL GROWTH FACTOR RECEPTOR (MEGF-R). Enkhtsetseg Purev, D. W Cai, E. Miller, B. Birebent, R. Somasundaram, T. Mayer, and D. Herlyn, *Albert Einstein Med Ctr, Philadelphia, PA, Memorial Hosp of Burlington, Mt. Holly, NJ, and The Wistar Institute, Philadelphia, PA*

mEGF-R is expressed by carcinomas of the breast, ovary, lung and colon, and also by gliomas, but not by normal tissues. The mEGF-R is the result of an 801 bp deletion within the extracellular domain of normal EGF-R and is expressed both on the surface and in the cytoplasm of the tumor cells. Thus, mEGF-R is a potential tumor-specific target for B and/or T cells in active and passive immunotherapy against tumors. We have evaluated humoral and cellular immune responses to mEGF-R in eight breast cancer patients and three healthy donors. Four patients with tumors expressing mEGF-R developed mEGF-R-specific humoral immune responses and two of these patients also developed mEGF-R-specific cellular immune responses. None of the patients with mEGF-R negative tumors developed cellular immune responses, and only one patient developed antibodies to mEGF-R. None of the three healthy donors demonstrated mEGF-R-specific humoral or cellular immune responses. These studies demonstrate that breast cancer patients can immunologically recognize mEGF-R and suggest that enhancement of the immune responses may be possible by vaccination of the patients against mEGF-R. (Supported by DAMD17-96-1-62 37 from the U.S. Department of Defense.)

#4444 IDENTIFICATION OF POTENTIAL CD4+ T HELPER EPITOPES DERIVED FROM THE PROTEIN SEQUENCE OF PROSTATIC ACID PHOSPHATASE (PAP). Douglas G McNeel, Lan D Nguyen, and Mary L Disis, *Univ of Washington, Seattle, WA*

PAP is a tumor antigen in prostate cancer. Vaccine studies in rodent models indicate cytotoxic T cells (CTL) are critical for generating destructive prostatitis. Consequently, vaccine trials targeting PAP, planned or in progress, are using strategies to maximize the induction of PAP-specific CTL. Several HLA-A2 peptides derived from PAP have already been identified. Immunization with HLA-A2 peptides alone may not be sufficient; the generation of long-lasting CTL responses may require T cell help. Investigations in a variety of models suggest that the addition of T help specific for the immunizing antigen may be effective in generating a robust antigen-specific CTL response. The purpose of this study was to identify potential T helper epitopes derived from PAP for inclusion in peptide-based prostate cancer vaccines. Standard algorithms were used to scan the PAP amino acid sequence for peptides likely to bind human MHC class II molecules. Ten 15- to 18-mer oligopeptides were chosen, constructed, and used as stimulator antigens in proliferative T cell assays using peripheral blood mononuclear cells derived from patients with (n=7) or without (n=13) previously identified T cell responses to PAP protein. Only rare peptide-specific T cell responses were detected in patients with no preexistent PAP protein response. Three peptides tested, however, generated responses in patients with preexistent PAP-specific T cell immunity significantly different from the PAP non-immune population. One of the peptides elicited an immune response in 6/7 PAP-immune patients tested and none of the PAP non-immune patients. This peptide may represent a universal T helper epitope for inclusion in PAP specific peptide-based vaccines.

#4445 IDENTIFICATION OF TUMOR ENDOTHELIAL CELL-ASSOCIATED ANTIGENS WITH PHAGE DISPLAY TECHNOLOGY. H. J Bloemendal, A. van Wolfswinkel, M F B G Gebbink, T. Logtenberg, and E. E Voest, *Univ Med Ctr and Utrecht Biotech Systems, Utrecht, Netherlands*

Angiogenesis is a critical step in the progression of tumors from dormancy to a clinically relevant cancer. Inhibition of angiogenesis and vascular targeting are considered new promising anti-cancer therapies. In order to inhibit neovascularization or target the tumor's vasculature it is pivotal to find antigens which are exclusively present on tumor endothelial cells (TECs) and not on endothelial cells of the normal vasculature. We have employed a large phage antibody display library of human single chain Fv (scFv) fragments to isolate scFv against freshly-isolated TECs. The phage library was incubated with single cell suspensions prepared from tumors of patients with renal cell carcinoma and TECs and attached phages were subsequently isolated by cell sorting using the endothelial cell marker Ulex europaeus agglutinin I. After four selection rounds, monoclonal single chain antibody fragments (scFv) were isolated and used in immunohistochemical and flow cytometric analysis. These studies yielded a scFv that recognizes the tumor vasculature in breast, lung and renal carcinoma, whereas adjacent, normal tissues were negative. In flow cytometric analysis, this scFv bound to freshly-isolated TECs but not to cultured human umbilical vein or dermal microvascular endothelial cells. In addition to the TEC's, the scFv only recognized peripheral human B-lymphocytes. Characterization of the epitope recognized by the scFv is in progress. Our study shows that phage display technology is a powerful tool to discover epitopes on TECs that may be assocaited with novel molecuIs. These scFv may be used to identify the gene encoding the target molecule and re-formatted to intact human monoclonal antibodies for targeting the tumor vasculature.

#4446 TARGET CELL KILLING BY GENETICALLY ENGINEERED PRIMARY T-CELLS EXPRESSING AN ANTIGEN-SPECIFIC CHIMERIC RECEPTOR. Peter Bernhard Dall, Bettina Durst, Gerd Bauerschmitz, Dieter Niederacher, and Hans G Bender, *MolGenLab, Ob/Gyn, Univ Med Ctr, Duesseldorf, Germany, Ob/Gyn, Duesseldorf, Germany, and Ob/Gyn, Univ Med Ctr, Duesseldorf, Germany*

Different types of cancer cells preferentially express variant epitopes of the CD44v-hyaluronate receptor family on the cell surface. In former studies it could be demonstrated that transfer of a gene encoding the TCR-ζ-chain and a CD44v-specific single-chain-antibody (scFv) into a cytotoxic T-cell line leads to antigen-specific killing of CD44v-expressing target cells. The following questions were: 1. gene transfer efficiency of primary lymphocytes, 2. receptor surface-expression and 3. CD44v-mediated killing. To evaluate the transduction efficiency immediately after the gene transfer a gene coding for a myc-tag was inserted between anti-CD44v7/8-scFv-gene and CD8α-spacer-gene. The complete construct included genes for the variable domains of heavy and light chain of the CD44v7/8-specific antibody, for the myc-tag, for the CD8-α-spacer and for the TCR-ζ-chain as T-cell-activation domain. This gene was introduced into a retroviral pLXSN-vector. T-cells isolated from the mouse spleen were cocultivated with the retrovirus-producing packaging line ΩE. Gene transfer rates of >% have been reached. Anti-myc-FACS-analysis of the infected T-cell pool documented surface expression of the chimeric receptor. Cytotoxicity data show killing of CD44v7/8-expressing target cells of up to 45%, depending on the effector/target-ratio. These data show that retargeting of primary T-cells towards a defined antigen induces antigen-specific T-cell cytotoxicity.

#4447 MODIFICATION OF THE TUMOR ANTIGEN GP2 IMPROVES INDUCTION OF GP2-REACTIVE CYTOTOXIC T LYMPHOCYTES. Yoshiyuki Tanaka, Peter S Goedegebuure, and Timothy J Eberlein, *Washington Univ Sch of Medicine, St. Louis, MO*

GP2 (IISAVVGIL), the p654-662 HER2/neu derived tumor antigen, induces HLA-A2 restricted cytotoxic T lymphocyte (CTL) reactive to various epithelial cancers. However, the binding affinity of GP2 to HLA-A2 has been known as very low. To improve the immunogenicity of GP2, we introduced ten different amino acid substitutions into GP2 at anchor positions. Four out of ten modifications, especially phenylalanine at position 1 (1F) based modifications, showed significant improvement of their binding affinity, which was almost equal to modified gp100 (G9-209 2M), using the T2 stabilization assay. These peptides were used to stimulate peripheral blood lymphocytes from HLA-A2 healthy donors using peptide-pulsed autologous dendritic cells (DC). After 3 times weekly stimulations, CTL activity against GP2 pulsed T2 (T2GP2) and SKOVA2 (SKOV3 HLA-A2 transfectant) was measured in ^{51}Cr release assays. Several modifications significantly enhanced GP2-specific CTL activity. In particular, Phenylalanine at position 1and Leucine at position 2 (1F2L) based modifications maximized the CTL activity against both T2GP2 and SKOVA2 (about 2.7 fold increase at E/T 40 against SKOVA2). IF2L based modifications increased not only the binding affinity to HLA-A2 but also improved immunogenicity of GP2. These data suggest that DC + modified GP2 may improve immune theraples for the treatment of HER2/neu overexpressing tumors.

cell lines. However, immunohistochemical analysis of prostate tumor tissue sections reveals nests of epithelial cells staining positive for Clar1. This staining is cytoplasmic, occurs predominantly in the epithelium, but does not occur in all malignant glands within a section. Quantitation of Clar1 staining reveals a positive correlation between the number of glands stained and prostate tumors of later stage and higher grade. This is consistent with our findings at the transcript level. Since Clar1 contains consensus sequences for SH3-binding, we are performing yeast two-hybrid analysis using a pEG202 "bait" vector containing 51 amino acids encompassing this region. We are using a HeLa cell cDNA library to identify clones that may interact with Clar1 in this system. The identity of any interacting clones and the possible role of Clar1 in signal transduction pathways will be discussed.

IMMUNOLOGY/EXPERIMENTAL AND PRECLINICAL 10: Vaccines and Tumor Antigens I

#3660 The CM101 Target Protein HP59 is a Pathoangiogenic Marker with Potential as a Vaccine and Drug Target. Barbara D. Wamil, Yufen Wang, Fenglei Sun, He-Ping Yan, and Carl G. Hellerqvist. *Vanderbilt University, Nashville, TN.*

CM101 is an anti-pathoangiogenic agent derived from the neonatal pathogen Group B Streptococcus (GBS). CM101 targets only embryogenic and pathologic vasculature in humans and mice. Preclinical and clinical studies have shown that CM101 binds rapidly to tumor vasculature, activates complement and initiates an inflammatory response, which leads to infiltration of the tumor of activated leukocytes and tumor apoptosis. We expression-cloned a target protein for CM101 and identified a seven transmembrane domain, highly conserved, glycoprotein named HP59 of approximate 63kD in its natural glycosylated form. Antibodies to peptides synthesized based on the amino acid sequence at the N-terminal end were generated and used to demonstrate and confirm that CM101 is exclusively expressed in human and mouse pathologic vasculature. We *hypothesized* that mammals immunized with HP59-derived peptides would show reduced rate of tumor growth. Male and female C57 mice were immunized three times with a mixture of five KLH conjugated peptides derived from the homologous proteins HP59 and SP55. Animals were bled after 4 weeks and shown to have antibody titers at 1:200 with OD of >2.0 to one of the extracellular peptides based on a 7TMD configuration for HP59. Lewis Lung cell suspensions (5 x 10^4 cells) in 3% agar were implanted i.d. in seven immunized male and five female mice and in four male and female controls immunized with adjuvant alone. The mice were observed daily and tumor volumes recorded every other day until the control tumors began to ulcerate at which time the mice were euthanized. Tissues, including tumor, were collected for histological analyses and IHC for HP59, CD31, and CD34 to map the angiogenic status of the tumor. The *results* showed that the tumor growth rate in the immunized mice was inhibited by 64% and a paired t-test analysis of data from the five last recordings of tumor volumes gave p=0.025. Immunized mice showed high titers of anti-HP59 antibody. Histological examination showed no evidence of toxicity to normal organs and IHC analysis showed a marked absence of HP59 positive tumor vasculature in the smaller tumors of the immunized mice. *Conclusion.* Immunization with the peptides derived from the pathoangiogenic marker, HP59, had no adverse effect on the mice, male or female, and resulted in a significant attenuation of the growth of the aggressive Lewis Lung tumor implant of 50,000 cells. Immunization is anticipated to protect against smaller tumor challenges, which will further validate HP59 as a pathoangiogenic target with great therapeutic potential.

#3661 Cancer Vaccines Targeting MN/CA IX Antigen for Renal Cell Carcinoma. Hirotsugu Uemura, Kazuhiro Shimizu, Masaki Cho, Eijiro Okajima, Yoshihiko Hirao, Shinsuke Saga, and Kazuhiro Yoshikawa. *Aichi Medical University, Aichi, Japan, and Nara Medical University, Nara, Japan.*

MN/CA IX (MN) antigen is a membranous glycoprotein expressed in a large number of renal cell carcinomas (RCC) but not in normal renal tissues. This antigen would be a therapeutic target for active specific immunotherapy in RCC. The aim of this study is to generate MN antigen peptide vaccines that have capacity to induce specific immunity against RCC. We have identified 9 mer peptides in MN antigen with binding motif for possible H-2K^d epitopes. To develop syngeneic system, MN-RenCa and MN-3T3, MN/CA9 transfectant mouse RCC (RenCa) and embryofibroblast cell lines (BALB-3T3) originated from BALB/c mouse, have been established. BALB/c mice were immunized with the antigen expressing cells or antigen peptides with IFA every week. After the fourth vaccination, MN specific CTL activity was tested against MN-3T3 and peptide-pulsed BALB-3T3 cells. Spleen cells derived from the immunized mouse showed specific reactivity against MN antigen. From these cells, CD8+CTL were generated and showed specific cytotoxicity against MN antigen presenting cells, i.e., induction of MN/CA IX specific CTL clone. This CTL (5 x 10^6) were transferred into naive mice after inoculation of MN-RenCa cells in their flank. Four weeks after inoculation, significant tumor growth inhibition was observed in the treated mice compared to control mice. Moreover, therapeutic efficacy of vaccination with antigen peptides was investigated in syngeneic animal model. BALB/c mice were vaccinated s.c. with the antigen peptides every week. After the fourth vaccination, MN-RenCa cells (5 x 10^6) were inoculated in their flank. Four weeks after the cell challenge, mice were sacrificed and tumor-take and size were determined. Vaccination with antigen peptides resulted in significant tumor growth inhibition compared to control groups. These finding suggest that our peptide vaccines derived from MN/CA IX antigen may be promising as cancer vaccines for the treatment of RCC.

#3662 Canine Melanoma Cell Line Transfected with Recombinant Human gp100 Elicits Cytotoxic T-Cell Response in Vitro. Andrew Nordstrom Alexander, Mike Huelsmeyer, and E. Gregory MacEwen. *University of Wisconsin-Madison, Madison, WI.*

Many tumor-associated antigens have been shown to be nonmutated self-proteins, which have differential expression on tumor cells in comparison with normal cells. An example of one such antigen that is expressed on 90% of those melanoma cells examined is glycoprotein 100 (gp100). Since gp100 is highly expressed on melanoma cells, it is an excellent candidate to be used as an immunotherapy designed to break "tolerance" to self-antigens, leading to in vivo cytolytic T-cell activity. In the current study we determined if canine melanoma cells have measurable levels of gp100 transcripts and if so, can gp100-specific immunity be enhanced using canine melanoma cells transfected with human gp100. Canine gp100 mRNA levels were determined using reverse transcription-PCR. Degenerative oligonucleotide primers were designed by aligning homologous regions from human, mouse, and horse gp100 sequences. Steady-state gp-100 transcripts were strongly expressed in melanocytic cell lines and weakly expressed in cells of non-melanocytic origin. We did not examine gp100 expression levels since an appropriate anti-canine gp100 antibody was not available. CML-I and 17CM98 canine melanoma cell lines were transfected with recombinant human gp-100 cDNA (hgp100) using Accell® particle mediated gene transfer. Following a 24-hour incubation, transfected cells were permeabilized, stained with anti-human gp100 HMB45 antibody, secondary GAM-FITC antibody, and analyzed by flow cytometry. A transfection efficiency of 7% in CML-I and 7.2% in 17CM98 canine melanoma cells were observed. In a co-culture system, hgp100-transfected cells were used to stimulate naïve canine blood mononuclear cells in vitro. These primed CTL's were shown to be cytotoxic for canine CML-1 melanoma cells, whereas effector cells co-cultured with non-hgp100 expressing cells, failed to elicit a CTL response. These results suggest that canine melanoma cells transfected with human gp-100 cDNA express high levels of human gp-100. More importantly, canine melanoma cells transfected with human gp-100 elicit specific anti-gp100 antitumor immunity and may constitute a potential immunotherapeutic strategy for tumor vaccine design in patients with established melanomas.

#3663 Transduction and Expression of the Human Carcinoembryonic Antigen Gene in a Murine Squamous Carcinoma Cell Line. Erik S. Kass, Vanessa Muniz-Medina, and Carter Van Waes. *National Institutes of Health, Bethesda, MD.*

Carcinoembryonic antigen (CEA) is a well-characterized oncofetal glycoprotein whose overexpression by human carcinomas makes it a rational target for cancer immunotherapy. Currently, CEA is being studied in preclinical animal studies and clinical trials as a target for specific immunotherapy of adenocarcinoma. Recently, we demonstrated the over-expression of CEA in cell lines derived from several head and neck squamous cell carcinomas (HNSCC). These results suggested that HNSCC may be considered as a possible candidate for immunotherapy. To our knowledge, murine squamous carcinoma cells do not express CEA. In the present study, a cell line derived from the mouse squamous cell carcinoma, Pam 212, was transduced with a retroviral construct containing complementary DNA encoding the human carcinoembryonic antigen (CEA) gene. Pam 212 is a spontaneously transformed cell line derived from keratinocytes of BALB/C mice. Two tranduced clones were positive for CEA by nested rT-PCR. These clones, termed Pam 212-cea4 and Pam 212-cea9, were also found to express high levels of CEA on the cell surface. The levels of CEA produced were comparable to those found in several human squamous cell carcinoma lines. Further analysis of cell lysates by western blot demonstrated that both clones expressed a single Mr 70,000 immunoreactive protein product. Several CEA-specific monoclonal antibodies were found to react with both clones as determined by FACS analysis and immunohistochemical staining. These results provide a basis for future studies to elucidate immunodominant epitopes of CEA in squamous cell carcinoma and to develop a syngeneic model system that may aid in the design of reagents and protocols to study immunotherapeutic strategies directed against squamous cell carcinomas expressing human CEA.

#3664 Synthetic Peptide Mimotopes Reacting to Single Chain Antibodies (scFv) Directed Against Tumor Rejection Antigen gp96 as Novel Immunogens. Ashok Badithe, Yuan-gen Chen, Abraham Mittelman, and Raj K. Tiwari. *New York Medical College, Valhalla, NY.*

Tumor derived purified preparations of heat shock protein, gp96, induces protective immunity in experimental prostate cancer animal models. Specificity of the tumor protective effect is conferred by the peptides associated with gp96. Identification of these tumor associated peptides can result in novel immunogens that mediate tumor rejection. We utilized the synthetic combinatorial single chain phage display antibody (scFv) library to differentially select out tumor specific peptides using tumor and non-tumor derived gp96. An array of phages that displayed scFvs that specifically reacted to tumor derived gp96-peptide complexes were selected and used to immunoprecipitate tumor associated protein(s). The identity of these phages containing scFv was established by sequence analysis. These experiments validate the hypothesis that scFvs can be used to identify specific tumor derived peptide (s)/protein antigen (s) and that heat shock proteins are a useful source of tumor associated antigens. The observed tumor specific reactivity of the scFvs to purified gp96, was directed either to the tumor associated peptides or tumor derived gp96-peptide complexes. The tumor specific single chain antibodies were used to screen a 12-mer (LX-8) or 15-mer (X-15) synthetic peptide library to generate synthetic peptide mi-

motopes that can potentially mimic the tumor rejection properties of gp96-peptide complexes. Using this strategy, several such synthetic peptides have been identified and their sequence analysis with BLAST searches in the protein data base did not show sequence homology. These peptides are now being actively characterized for their prophylactic and therapeutic effects on both primary cancer growth and dissemination of metastasis in prostate cancer. (Funded by the US ARMY Grant no. 17-98-1-8534)

#3665 Inhibition of PSMA-Positive Tumor Growth by Vaccination with Either Full-Length or the C-Terminus of PSMA. Richard F. Jones, Katsuyuki Kuratsukuri, Nobuyasu Nishisaka, Philo Morse, Gabriel P. Haas, and Ching Y. Wang. *SUNY Upstate Medical University and VA Medical Center, Syracuse, NY.*

Adaptive immune responses against malignant tumors induced by vaccination with selected tumor-associated antigens can be therapeutic. For immunotherapy of prostate cancer, we used a model system to study immunization with defined regions of the extracellular domain of prostate specific membrane antigen (PSMA), a surface antigen expressed by most prostate cancer cells. The anti-PSMA vaccines used in this study were NIH3T3 cells cotransfected with pST/neo plus pEF-BOS expressing either full-length 750 amino acid PSMA or only the C-terminal 180 amino acid region (PSMAc). This region lies beyond the transferrin receptor-like sequence in the extracellular domain of PSMA, which reportedly internalizes upon antibody binding. Expression of recombinant PSMAs by 3T3 cells was confirmed by immunohistochemical (IHC) staining and/or RT-PCR. The control vaccine was 3T3/neo, which was transfected with pST/neo alone. 3T3/neo was negative for PSMA expression. For experimental target tumors, murine renal-cell carcinoma cells (Renca; isogenic with Balb/c) expressing recombinant full-length human PSMA were injected subcutaneously into Balb/c mice. For vaccination, 10 million irradiated vaccine cells (2000 rads) were injected intraperitoneally every week for 4 weeks. One week later, 1 million Renca/PSMA tumor cells were injected subcutaneously. In control mice, which were vaccinated with 3T3/neo or vehicle alone, tumors were palpable by ~13 days and lethal by 5 weeks. In contrast, tumor growth was inhibited >50% and survival was substantially increased in mice vaccinated with 3T3/PSMA or 3T3/PSMAc, while PSMA-negative tumor growth was unaffected in these animals. Serum collected from mice 4 weeks after injection with either 3T3/PSMA or 3T3/PSMAc was able (at 1:20 dilution) to intensely IHC stain the PSMA-positive human prostate cancer line LNCaP, but not control cells. This indicated that a strong Th2 host response to both PSMA and PSMAc correlated with anti-tumor activity. In contrast, serum from mice vaccinated with 3T3/neo gave only background staining of LNCaP cells. These results indicate that the 180 amino acid C-terminal domain of PSMA may have potential for immunotherapy of prostate cancer. Since antibodies against other regions of PSMA reportedly lack anti-tumor activity, PSMAc-directed antibodies may have the advantage of not internalizing upon antibody binding, so may thus support antibody dependent cell-mediated cytotoxicity. Supported by a VA Merit Review grant.

#3666 MYCN Amplified and Multidrug Resistant Neuroblastoma Cell Lines Are Killed by CD8+ T Lymphocytes Directed Against MYCN Peptides. Hong-Wei Wu, Leonid Metelitsa, Mark Podberezin, and Robert C. Seeger. *Childrens Hospital Los Angeles, Keck School of Medicine, USC, Los Angeles, CA.*

Amplification and overexpression of the proto-oncogene MYCN occurs in 33% of metastatic neuroblastomas, and this correlates with rapid tumor progression and poor outcome of patients. Because the MYCN protein is highly expressed, it might serve as a potential target for active or adoptive T-cell immunotherapy. We have HLA genotyped 21 human neuroblastoma cell lines and identified 7 that are HLA-A1 (7 MYCN amplified), 8 that are HLA-A2 (5 amplified, 1 non-amplified, 2 unknown), and 6 that are HLA-A24 (5 amplified, 1 non-amplified). This panel of cell lines, which were derived before or after clinical disease progression includes those resistant to cisplatin, carboplatin, etoposide, doxorubicin and/or melphalan. Using an Applied Biosystems Model 430A peptide synthesizer, we synthesized peptides with predicted binding to HLA A1 (n=3), HLA A0201 (n= 8), and HLA A24 (n= 3). Three HLA-A1 specific peptides have been tested for their ability to generate CD8+ cytotoxic T lymphocytes (CTL) directed against them. Monocyte-derived dendritic cells were generated from peripheral blood with GM-CSF and IL-4 and then matured with TNFa. CD8+ T cells that were isolated with magnetic immunobeads from the same normal donors were stimulated weekly for 4 cycles by autologous mature dendritic cells pulsed with peptides to generate MYCN specific CTLs. Cytotoxic activity was tested against MYCN amplified neuroblastoma cell lines (drug sensitive and resistant) using calcein AM labeled target cells and measuring residual calcein fluorescence by digital image microscopy scanning. An HLA A1 peptide (STMPGMICK) generated CD8+ lymphocytes (98.5% CD8+) that killed MYCN amplified and multidrug resistant CHLA 134 HLA A1 cells (cell viability = 65.0 ± 3.5%) but not IMR-32 HLA A2 cells (cell viability = 89.2 ± 2.5%; p = 0.0001). We conclude that MYCN amplified neuroblastoma cells that are overexpressing the protein can be killed by CD8+ CTL that recognize HLA class I restricted MYCN derived peptides. Of importance, this cytotoxicity can be effective against multidrug resistant neuroblastoma cells.

#3667 Impact of p53-based Immunization on Chemical Induced Carcinogenesis in Mice: Differences in the Pattern of p53 Exon 7 Mutations Expressed in Tumors Induced in Immunized Mice Relative to Those Induced in Control Mice. Vito R. Cicinnati, Grzegorz Dworacki, Susanne Beckebaum, Andreas Albers, Saroj Sigdel, Thomas Tueting, and Albert DeLeo. *University of Mainz, Mainz, Germany, and University of Pittsburgh Cancer Institute, Pittsburgh, PA.*

The targeting of wild-type sequence (wt) p53 epitopes represents a potential basis for a broadly applicable cancer vaccine. As a preclinical murine tumor model system for p53-based immunotherapy of cancer, we are studying the impact of p53 vaccines on the induction of chemically induced sarcomas in mice, nearly all of which express altered p53 molecules. In these experiments, before and after being challenged with methylcholanthrene, groups of ten CB6F1 mice each were immunized with vaccines targeting the CTL-defined, H2-Kd-restricted wt p53 232-240 epitope. This epitope is encoded by p53 exon 7. The vaccines used consisted of peptide-pulsed DC or pCI-ES57 DNA; the latter a non-viral plasmid vector construct expressing wt p53 232-240 linked to a class I presentation enhancing signal (ES) sequence. It was delivered biolistically using DNA coated gold particles. Analysis of the p53 exon 7 status and H2-Kd phenotype of early passage cell lines (<3) derived from the tumors induced in immunized mice is revealing a decrease in the proportion of tumors capable of presenting the wt p53 232-240 epitope relative to that of control tumors. Over half of the 14 analyzed controls have the capacity to present the epitope; 8 are wt exon 7 and H2-Kd+. The remaining 6 tumors do not; 4 have mutations within the epitope and 2 have flanking mutations at codons 241 or 243 (a p53 mutation at flanking codon 273 has been shown to block the processing of the human p53 264-272 epitope). Of the 6 tumors induced in p53 232-240/DC-immunized mice analyzed to date, only one has a reasonable potential to present the epitope (wt exon 7 and H2-Kd+). The remaining 5 tumors have little to no potential for presenting this epitope due to mutations either within or flanking the epitope; 2 are wt exon 7 but H2-Kd-, one has a mutation within the epitope and the other 2 have mutations in codon 246. Of the 8 tumors induced in pCI-ES57-treated mice analyzed, none appear capable of presenting the epitope. Four express wt exon 7 but little to no H2-Kd, and the other four have mutations within the epitope or flanking it at codons 241 or 245. As the majority of the tumors induced in immunized mice, appear to have avoided immune detection either by downregulation of class I MHC expression or mutation within or flanking the targeted p53 epitope, it suggests that the p53 vaccines promoted the outgrowth of "epitope-loss" tumors. These results have serious implications for p53-based immunotherapy of cancer and warrant further studies aimed at avoiding outgrowth of "p53 epitope-loss" tumors.

#3668 Preclinical Testing of a Peptide-based, HER2/Neu Vaccine in Prostate Cancer. Gayle Blanchard Ryan, Brian A. Fisk, Constantine G. Ioannides, Isabell A. Sesterhenn, Judd W. Moul, David G. McLeod, and George E. Peoples. *Armed Forces Institute of Pathology, Washington, DC, UTMD Anderson Cancer Center, Houston, TX, and Walter Reed Army Medical Center/Uniformed Services University of the Health Sciences, Washington, DC.*

The HER2/neu protein is over-expressed in multiple epithelial tumors and is the source of immunogenic peptides currently under investigation in vaccine trials in ovarian and breast cancers. To determine the potential efficacy of this vaccine strategy in prostate caner, we have screened 12 patients for HER2/neu expression by immunohistochemistry after prostatectomy. These patients were predicted to have a high (n=7) or intermediate (n=5) risk for recurrence based on biostatistical modeling. In the intermediate risk group, 20% stained positive; however, in the high risk group, 100% over-expressed HER2/neu (P=0.004). With this target population identified, the immunogenicity of the HER2/neu peptide, E75 (KIFGSLAFL), was investigated. Cytotoxic T-lymphocytes (CTL) were isolated from HLA-A2+ healthy donors and prostate cancer patients. Healthy donor CTL stimulated in vitro with E75 (weekly x 3) specifically recognized T2/E75 in both standard cytoxicity assays (14-23% specific lysis at an E:T=6:1) as well as IFN-γ release assays. E75-specific CTL were also shown to have cytotoxic effect on HER2/neu+, HLA-A2 + tumor cells. More importantly, prostate cancer patients' CTL recognized T2/E75 after a single stimulation, with higher killing (21-34%) and at a lower E:T ratio (3:1) compared to healthy donor CTL. This finding suggests that prostate cancer patients have a higher CTL precursor frequency from in vivo sensitization to HER2/neu. Furthermore, the patients' E75-specific CTL recognized HER2/neu +, HLA-A2 + tumor cells with cytotoxic levels 3.5 fold higher than the healthy donor CTL. In conclusion, prostate cancer patients at high risk for recurrence have a high level of HER2/neu expression. This may indicate that HER2/neu status is a prognostic factor. The HER2/neu peptide, E75, is immunogenic in healthy donors and even more so in prostate cancer patients. Based on these findings, a Phase I/II clinical trial has been initiated evaluating the HER2/neu peptide as a preventive vaccine targeting prostate cancer patients at high risk for recurrence.

#3669 Immunogenicity and Specificity of HLA-A2.1-Restricted Peptides from Carcinoembryonic Antigen (CEA) and Nonspecific Cross-Reacting Antigen (NCA) in Transgenic Mice. Hajime Tanaka and F. James Primus. *Vanderbilt-Ingram Cancer Center, Nashville, TN.*

The purpose of this study was to compare the immunogenicity of closely-related HLA-A2.1-restricted CEA peptides in transgenic mice. Peptides selected for study shared a common 6-mer motif and possessed a dominant anchor residue at position 9 or 10. Our previous studies that utilized human breast carcinoma cells demonstrated by ESI-MS/MS analysis that one peptide, CEA-5, was naturally processed (Protein Sci., 5:116, 1996). Four peptides from CEA and one from NCA were used to

associated cancer cells and investigated whether specific ET-1 antagonists may inhibit the autocrine growth of cervix cancer cells in vitro and in vivo. Cervical carcinoma derived cell lines (CaSki, SiHa and C33A) and spontaneous immortalized human keratinocytes (HaCaT cells) were utilised. All HPV positive cancer cells secreted ET-1 and expressed functional ETA receptor (ETAR) whereas a HPV negative carcinoma cell line expressed only the ETBR and didn't secrete ET-1. ET-1 stimulated a marked dose-dependent increase in [3H]-thymidine incorporation respect to the normal cells whereas ET-3 and ETB agonists had no effect. In HPV-positive cancer cells a specific antagonist of ETAR inhibited the proliferation induced by ET-1 and dramatically affected the basal growth rate of cervical tumour cells. The action of the specific ETA receptor antagonist ABT 627 was analysed in vivo by monitoring its therapeutic action on early and advanced stages of CaSki and C33A cervical carcinoma xenografts. In vivo ABT 627 (1mg/Kg/12h i.p. for 21 days) was able to reduce the tumor growth of CaSki cervical carcinoma xenografts. These experimental evidences demonstrate that ET-1 participates in the progression of neoplastic growth in HPV associated carcinoma, in which ET-1 and ETAR are overexpressed and could be targeted for antitumor therapy. The in vivo results indicate that the ETAR antagonists may provide a novel approach to the treatment of cervical carcinoma. Supported by AIRC, FIRC, CNR Biotechnology Project and Ministry of Health

#4147 Tumor Cells Transduced to Secrete an Angiopoietin Decoy Show Delayed Tumor Take, Impaired Neo-Angiogenesis and Increased Leukocyte Infiltration in Synergy with rIL-12. Cecilia Melani, Chiara Foroni, Antonella Stoppacciaro, Alessandra Caré, and Mario P. Colombo. *Istituto Nazionale Tumori, Milan, Italy, Istituto Superiore di Sanità, Rome, Italy, and University La Sapienza, Rome, Italy.*

The endothelial-specific thyrosine kinase receptor, Tie2/TEK is expressed predominantly on endothelial cell precursors and is necessary during the process of sprouting and branching and/or remodelling that occurs upon VEGF stimulation of neo-angiogenesis. Angiopoietin-1 is expressed in proximity of developing blood vessels, and its receptor, Tie2/TEK, has been detected on endothelial cells of vessels penetrating growing tumors. Moreover, a soluble form of Tie2/TEK receptor has been shown to inhibit tumor growth by blocking neo-angiogenesis. The extracellular domain of the receptor tyrosine kinase Tie2/TEK (exTEK) has been used as an angiopoietin decoy to test the role of angiopoietins in the organization of neo-vessels at site of tumor growth and metastatization. C-26 adenocarcinoma cell line secreting detectable amount of exTEK were able to inhibit tube formation by HUVEC cells plated on a growth factor-reduced Matrigel. In vivo C26TEK displayed reduced ability to form either lung or liver experimental metastases, that eventually grew with a significant delay. The delay in metastatic take occurred in normal as well as in athymic hosts. Immunohistological analysis of lung metastasis revealed increased leukocytic infiltration in C26TEK compared to C26, and signs of inflammatory activation. Accordingly, tumor associated blood vessels expressed higher level of VCAM-1 and ELAM-1 and showed impairment in endothelial cell growth and organization. A quantitative estimation of impaired endothelial growth in C26TEK was obtained by injecting transduced cells s.c. into CVE-CAT mice, a transgenic strain carrying the CAT gene under the VE-cadherin promoter. Leukocyte infiltration participates in impairing the take of lung metastases and the growth rate of s.c. tumors likely through different mechanism since rIL-12 synergizes with exTEK in s.c. tumors but not in lung metastases. These data while confirming the activity of soluble TEK as decoy receptor, underlines the role of inflammatory cells as co-modulator of tumor associated neo-angiogenesis.

#4148 *In Vitro* and *in Vivo* Evaluation of $^{111}In/^{90}Y$ Radiolabeled Peptides for Specific Targeting of Tumors Expressing Gastrin Releasing Peptide (GRP) Receptors. Timothy J. Hoffman, Charles Smith, Hariprasad Gali, Nellie Owen, Gary Sieckman, and Wynn Volkert. *U.S. Department of Veterans Affairs, Columbia, MO, and University of Missouri - Columbia, Columbia, MO.*

Gastrin releasing peptide receptors (GRP-R) have been shown to be present in a variety of tumors of neuroendocrine origin including prostate, pancreatic, and small cell lung cancer. We have synthesized a series of GRP-R specific peptides incorporating a DOTA radiometal chelation system suitable for complexation with ^{111}In, ^{90}Y, as well as the radiolanthanides. The ^{111}In and ^{90}Y complex of one lead candidate, DOTA-8-Aoc-BBN[7-14]NH_2 has been synthesized and evaluated *in vitro* and *in vivo*. *In vitro* competitive binding assays, employing PC-3 human prostate tumor cells, demonstrated an average IC_{50} value of 1.69 nM for the ^{111}In-DOTA-8-Aoc-BBN[7-14]NH_2 complex. *In vivo* pharmacokinetic studies of ^{111}In-DOTA-8-Aoc-BBN[7-14]NH_2 in PC-3 prostate tumor bearing mice conducted at 1,4,24,48, and 72 hrs p.i. revealed efficient clearance from the blood pool (0.92 ± 0.58 % ID, 1 hr p.i.) with excretion through the renal and hepatobiliary pathways (87% ID and 8.5% ID, at 24 hrs p.i., respectively). Initial tumor uptake of 3.63 ± 1.11% ID at 1 hr p.i. was observed with 49% and 43% retention at 4 and 24 hrs p.i., respectively. Similar pharmacokinetic properties were observed with ^{90}Y-DOTA-8-Aoc-BBN[7-14]NH_2. Initial therapeutic assessment of the ^{90}Y complex in PC-3 xenografts demonstrated that radiation doses of up to 20mCi/kg were well tolerated with overall survival exhibiting a dose dependent response. These pre-clinical observations suggest that peptide conjugates of this type may exhibit properties suitable as clinical diagnostic/therapeutic radiopharmaceuticals.

#4150 Synthetic Peptide Antigen Mimics to HER-2/neu. Ashok T. Badithe, Linda David, Bani Chander, Abraham Mittelman, and Raj Tiwari. *Johns Hopkins University, Baltimore, MD, and New York Medical College, Valhalla, NY.*

HER-2/neu, an oncoprotein overexpressed in human breast cancer is a target for therapeutic approaches. Since HER-2/neu is a self-protein most breast cancer patients develop tolerance and do not elicit an immune response. The objective of our present study was to develop synthetic peptide mimotopes to HER-2/neu that could break self protein tolerance. We used two synthetic phage display peptide libraries, LX-8 consisting of 12-mer peptides and X-15 consisting of 15-mer peptides and panned the libraries against three different monoclonal antibodies, Ab2, Ab4 and Ab5. All of these antibodies were against the extracellular domain of the HER-2/neu protein. Panning over each of the antibodies was undertaken and ten to hundred fold enrichment was noted after each round of panning. Distinct peptide clones reacting against the specific antibodies were obtained. We obtained twelve peptides that specifically reacted to Ab2 and did not react to either Ab4 or Ab5. DNA sequence analysis of the twelve clones revealed eight distinct clones that did not shown any appreciable sequence homology on BLAST searches with known protein data bases. We also analyzed the phage clones that react to Ab4 and Ab5. Reactivity of phage clones from either library to these antibodies was at least two to ten fold lower than the phage clones reactive to Ab2. Some of the phage clones that were panned over Ab4 or Ab5 also reacted to Ab2 suggesting a phenomenon of immunodominance of epitopes as Ab2 reactive clones do not react to Ab4 or Ab5. Several of the Ab2 reactive peptides in a concentration dependent manner inhibited the binding of Ab2 to HER-2/neu suggesting that in vitro these synthetic peptides could antigenically mimic HER-2/neu epitopes. The ability of these synthetic peptides to break tolerance in vivo and to induce an immune response to HER-2/neu in transgenic experimental animal models is being examined. (Funded by the US ARMY Grant # 17-98-1-8534, Zalmin A. Arlin Cancer Fund, Breast Cancer Alliance Fund)

#4151 Targeted Therapy of Solid Tumors with a Novel Ep-CAM Specific Immunotoxin Obtained by Fusion of Pseudomonas Exotoxin a to the High Affinity scFv 4D5MOC-B. Max Posch, Robert A. Olie, Martina Müller, Jörg Willuda, Suzanne Kubetzko, Andreas Plückthun, Rolf A. Stahel, and Uwe Zangemeister-Wittke. *Institute of Biochemistry, University of Zurich, Zurich, Switzerland, Schering AG, Berlin, Germany, and University Hospital Zurich, Zurich, Switzerland.*

The aim of our study was the development and preclinical testing of a novel recombinant immunotoxin with high potency and specificity for solid tumors. The epithelial cell adhesion molecule (Ep-CAM) is abundantly expressed in many solid tumors and represents a promising target for antibody-based therapies. The Ep-CAM specific high affinity single-chain antibody fragment (scFv) 4D5MOC-B was derived from the hybridoma MOC-31 by phage display, and its stability was improved by loop grafting of the complementarity determining regions onto the humanized framework of antibody 4D5 followed by the exchange of selected histidine residues in the VH core region. To employ the favorable tumor targeting properties of scFv 4D5MOC-B for therapeutic purposes it was fused with a truncated form of Pseudomonas exotoxin A (ETA) which lacks the natural cell binding domain I. The resulting Ep-CAM specific immunotoxin 4D5MOC-B-η was produced in E.coli and purified by metal-affinity and anion exchange chromatography. In vitro, 4D5MOC-B-η specifically inhibited protein synthesis in cells of Ep-CAM positive solid tumors of diverse histological origin and reduced cell viability with IC50 values ranging from 0.01 to 1 pM. In athymic mice, the systemic administration of 4D5MOC-B-η at a dose of 10 mg/day resulted in the regression of established tumor xenografts during the time of treatment, and showed anti-metastatic potential. This is the first report describing the potent anti-tumoral activity of a recombinant Ep-CAM specific immunotoxin which deserves attention for use in cancer therapy.

#4152 Growth Inhibition of Ewing's Sarcomas Following Treatment With Antityrosine Kinase Receptor Compounds. Susan A. Burchill, Jane Withey, and Jerry McMahon. *St James's University Hospital, Leeds, UK, and Sugen Inc, San Francisco, CA.*

Growth factors and their receptors are important in normal cell growth, activating signaling pathways, which can stimulate or inhibit cell division, differentiation and migration. Aberrant expression of these proteins can contribute to tumourigenesis by modulating tumour cell attachment, growth and angiogenesis. The aims of this study were to investigate whether growth factors and their receptors provide a growth advantage for tumours of the Ewing's sarcoma family (ES), and whether small molecule antigrowth factor receptor strategies might modulate their behaviour. Four ES cell lines were studied (TC-32; RD-ES, TTC-466 and SK-ES-1). Growth of these cell lines under normal, reduced and serum-free conditions was examined. A growth factor dependent neuroblastoma cell line (SK-N-SH) was included as a con-

ence in cytotoxic activity of NK-92 a... ...ced NK-92-scFv(FRP5)-ζ cells towards ErbB2 negative targets w... ...contrast, even at low effector to target ratios NK-92-scFv(FRP5)-... ...cifically and efficiently lysed ErbB 2 expressing tumor cells of vario... ...at were completely resistant to cytolytic activity of parental NK-92. 9... ...oB2-positive primary breast cancer cells isolated from pleural effusi... ...ents with recurrent disease were selectively killed by NK-92-scFv(FP... ...s. Our results demonstrate that efficient retargeting of NK-cell cyto... ...n be achieved, and might allow the generation of potent cell-based ther... ...s for the treatment of ErbB2 expressing malignancies.

#4797 Murine monoclonal anti-idiotype antibodies ... ...te antigens for 19A211, a sialylated carbohydrate antigen ass... ...th superficial bladder cancer. Nancy Frenette, Alain Bergeron, He... , and Yves Fradet. *Centre de recherche, Hotel-Dieu de Quebec, CHU ..., QC, Canada.*

Bladder cancer offers a unique opportunity to ... a cancer vaccines. Up to 75% of primary bladder tumors are superfi... ...nough treated effectively by transurethral surgery, recurrences occu... ...patients. Moreover, these tumors respond well to non-specific imm... ...y using BCG. Vaccines based on bladder tumor-associated antigens ...sed to prevent recurrence of this disease. Monoclonal antibody (mA... reacts with an antigen that is expressed on about 70% of superfi... ...tumors. We previously showed that mAb 19A211 reacts with a bl... ...er-associated sialylated carbohydrate epitope present on a group o... ...luding a glycoform of CEA. Its expression is restricted to tumor ce... ...exception of superficial umbrella cells of normal bladder in 25% ...uals. The aim of this study was to produce a murine monoclonal ... a antibody. BALB/C mice were immunized with mAb 19A211 conj... ...syhole Limpet Hemocyanin in presence of QUIL-A adjuvant. Hybrid... ...ction resulted in 1478 clones of which 26 reacted with 19A211 and ... ...ntrol antibodies. In inhibition assays, 14 of the 26 clones inhibited th... ...mAb 19A211 to its antigen expressed on MGH-U3 bladder cancer c... ...uggests that these antibodies can mimic the natural 19A211 antige... ...ential of these surrogate antigens to induce, in allogeneic mice and rab... ...response against superficial bladder tumors will be discussed.

#4798 Comparison of bispecific antibodies for pretargeted delivery of small molecules to tumors. Marianne K. Hayes, Hong M... Kim J. Consolino, Richard P. Tomko, Ching Y. Wang, Hans J. Hansen, Dav... Goldenberg, and Zhengxing Qu. *Immunomedics Inc, Morris Plains, NJ.*

Bispecific antibodies (bsAbs) with one specificity ... associated antigen and another for a hapten are employed in a ...o-step pretargeting system for radioimmunodetection (RAID) and r... therapy (RAIT). In this system, the radionuclides are directed to the ... by high-affinity binding of divalent hapten molecules to the tumor b... ...s. The radiolabeled hapten molecules by themselves, usually low ... weight peptides, are rapidly cleared from the blood and almost ev... excreted in the urine, resulting in little, if any, cytotoxicity to normal ... efficacy of this system is dependent on the pharmacokinetics o... ...s, including tumor penetration, non-tumor tissue retention and ble... ...ce. These properties are influenced by the structure and binding ... of the bsAbs. To determine the optimal configuration of bsAbs fo... RAIT, various forms of anti-CEA and anti-In-DTPA bsAbs were e... ...by genetic engineering and transfection. These bsAbs differ in their ...ncies to CEA and/or In-DTPA, and the ability to interact with Fc re... ...ir *in vivo* biodistribution and pretargeting properties were investigat... the bsAbs showed specific tumor targeting in mice bearing hum... tumors. The time needed for blood clearance (to a level lower than ...g) was proportional to the size of bsAbs, i.e. Fab-scFv (~75 kDa) < ... (~100 kDa) < IgG-$(scFv)_2$ (~200 kDa), at about 2, 3, and 7 days ...ly. A point mutation in the Fc domain that diminishes the inter... ...nR significantly accelerated the blood clearance of IgG-$(scFv)_2$ to 3-... ...umor uptake of the bsAbs with two binding sites for CEA was generally higher ...an that of monovalent bsAbs. In the pretargeting system, ^{99m}Tc-In-DTPA peptide was administered when a pre-set tumor-to-blood ratio of the pretargeted bsAb had been achieved (5-10 or higher). The radiolabeled peptide was specifically directed to tumor sites. BsAbs with two binding sites for In-DTPA directed more radiolabeled peptide to the tumor sites. However, trace amount of IgG-$(scFv)_2$ in the circulation trapped the In-DTPA peptide, resulting in poor tumor-to-non tumor ratios. The mutation in the Fc domain minimized the trapping and improved peptide targeting. Further studies are in progress to optimize the configuration of the bsAbs for tumor localization and peptide targeting. (Supported in part by SBIR grant CA81760 from the NIH to HJH.)

#4799 Single chain antibodies (scFv) isolated from a phage display library as a tool to identify unique gp96 associated peptide antigens. Ashok T. Badithe, Yuangen Chen, Abraham Mittelman, and Raj Tiwari. *New York Medical College, Valhalla, NY.*

Protective immunity by purified preparations of the heat-shock protein, gp96, is attributed to associated peptides. Isolation of these peptides has been a challenge, although tumor rejection property has been linked to the gp96-peptide complex as neither the peptides nor gp96 alone can confer protective immunity. We have been developing scFv reagents from a synthetic combinatorial phage display library that can identify the unique species of tumor associated gp96-peptide complexes in a complex pool of non-tumor and tumor specific gp96-peptide complexes. We utilized the VSV8 peptide, RGYVYQGL, and gp96 purified from a rat tumor to validate our hypothesis. The peptides at 1:50 molar ratio of gp96 to peptide were loaded on to purified preparation of gp96. In our experiments purified gp96 was not stripped of their tissue specific peptides. VSV8 peptide loaded gp96 and unloaded gp96 were used to differentially pan a synthetic combinatorial phage display antibody library. After four rounds of successive panning and amplification, we screened the soluble scFvs for reactivity to VSV peptide alone, VSV-peptide-gp96 complexes and gp96 alone. We isolated scFvs that reacted specifically to gp96-VSV peptide complexes and did not react to gp96 or peptide alone. These scFvs had distinct CDR3 sequences suggesting that different groups of antibodies can be generated that recognize specific sub-species of gp96-peptide complexes. We are currently extending these preliminary feasibility studies to utilize the scFvs from phage display antibody libraries to identify and better define the immunogenic subspecies of tumor specific gp96-peptide complexes.

#4800 Construction and characterization of bispecific minibody structures to enhance anti-tumor immune response. Lillian S. Shahied, Eva M. Horak, Heidi H. Simmons, and Louis M. Weiner. *Fox Chase Cancer Center, Philadelphia, PA.*

In recent years monoclonal antibody therapy has become a more popular and widely accepted treatment for a variety of diseases such as cancer. Antibody-dependent cellular cytotoxicity (ADCC) may be one of ... mechanisms by which clinically effective antibodies such as rituximab ar... stuzumab exert their therapeutic effects. In order to improve this anti-t... chanism of action, a series of bispecific antibodies was constructed ... two single-chain antibody fragments (scFvs) with different targets f... antigen HER2/*neu* and the leukocyte FcγRIII receptor) were joined b... amino acid linker. Several mutants were created with varying affinity ...R2/*neu* while linked to the same anti-FcγRIII scFv. The results fro... studies indicated that an increased affinity for HER2/*neu* correlated ...creased ability of the bispecific antibody to potentiate ADCC. It was ...med that activation of leukocytes did not occur in the absence of tur... gagement; this property minimizes unwanted leukocyte activation, ... ant host toxicity, in the absence of tumor engagement. However, ... tumor cell lysis observed using these novel scFv-based reagents w... ted when compared with the cytotoxicity seen with a full-length bispe... body. This diminution in activity could be due to the reduced wingspan ... of the $(scFv)_2$ molecules in comparison to a full-length IgG. Accordi... e creating bispecific antibodies that are larger and possess an incre... ility but still retain the same specificity. A bispecific minibody was ... d in which the IgG1 CH3 constant domain serves as the oligomerizatic... and is attached to the anti-FcγRIII and the anti-HER2/*neu* scFvs via ... amino acid linkers, respectively. This molecule can be expressed in ma... an cells from a dicistronic vector and has been purified using sequential ... ity purification techniques. Analysis by surface plasmon resonance shows that the bispecific minibody can bind to HER2/*neu* and FcγRIII, both individually and simultaneously. Furthermore, cytotoxicity studies show that the minibody can induce tumor cell lysis at 40 nM concentration to the same extent as a related IgG bispecific antibody. A trimeric, bispecific minibody has also been expressed using a strategy similar to the one employed to make the bispecific minibody. This construct is dimeric for binding to HER2/*neu* but binds monomerically to FcγRIII. The cytotoxic potential of this new construct is being compared to that of the bispecific minibody, bispecific $(scFv)_2$ and bispecific IgG.

#4801 Isolation and *in vitro* and *in vivo* characterization of human anti-human VEGF scFv fragments. Xinhui Wang, Jianming Hong, Yumi Yokoyama, Sunda Ramakrishnan, and Soldano Ferrone. *Roswell Park Cancer Institute, Buffalo, NY, University of Minnesota, Minneapolis, MN, and Roswell Park Cancer Institute, Buffalo, NY.*

Vascular endothelial growth factor (VEGF) stimulates proli... endothelial cells and contributes to the development of solid tum... noting tumor angiogenesis. Anti-VEGF monoclonal antibodies ... been shown to inhibit the functional activity of VEGF *in vitro* and ... transplanted with human tumors. These findings have provide... ground to implement clinical trials with anti-VEGF mAb in patier... gnant diseases. Potential limitations of the clinical application of ... mAb are the poor diffusion from the vasculature into the tumor ... organ antibody accumulation associated toxicity. These limitati... overcome by the application of single chain fragments of vari... (scFv) of antibodies. The latter offer also the advantage that the... ily modified genetically to increase their specific targeting to mal... . Therefore, we have isolated VEGF binding scFv fragments and ... ir functional properties *in vitro* and *in vivo*. Panning of a sem... phage display human-derived scFv library with recombinant h... o5 resulted in the isolation of 56 clones. Testing in a binding ass... 165 of the 56 clones identified 14 positive clones. DNA sequenc... 4 positive clones identified the same sequence in 13 clones and a ... in the remaining one. The latter clone, named JH2, and one of the 13 c... named JH1, were selected for additional studies. Soluble scFv JH1 and JH2 ...cted specifically in a standard sandwich assay with VEGF165 and VEGF121, which are the predominant isoforms of VEGF. Moreover, soluble scFv JH1 markedly neutralized VEGF165 and VEGF121 induced *in vitro* proliferation of

Proteasome inhibitors differentially affect heat shock protein response in cancer cells

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Abstract. The heat shock proteins (HSPs) are molecular chaperones that are emerging as biochemical regulators of cell growth, apoptosis, protein homeostasis and intracellular targeting of peptides. The immunological function of the HSPs are imparted by tissue specific peptides associated with the HSPs and as such autologous cancer derived HSP-peptide complexes are unique therapeutic agents. Since a majority of the intracellular peptides are generated by the proteasome, we examined the consequence of abrogation of proteasome function by proteasome inhibitors (PIs) such as Lactacystin, MG-132 and LLM on the growth and induction profile of HSP70 and gp96 using hematopoietic, lymphoid, and epithelial derived cancer cell lines. The effect on growth was measured by the XTT assay and induction of the heat shock proteins by Western blot analyses using HSP70 and gp96 specific antibodies. Of the PIs tested, cancer cells, were most sensitive to MG-132 and least sensitive to LLM. MG-132 also showed a 10-fold differential sensitivity between estrogen receptor positive, (ER+) MCF-7 cells and negative cells, (ER-) MDA-MB-231. Induction of heat shock proteins, gp96 and HSP70 was, however, noted in response to LLM. Since LLM exhibited minimal cytotoxic effect, metabolic stress that results in induction of HSPs may not be translated in cell growth inhibition and that there may exist a cell-type specific phenomenon in the HSP response to PI mediated metabolic stress.

Introduction

Heat shock proteins (HSPs) are a class of constitutive, well conserved class of proteins that respond to cellular stress. This stress is not restricted to elevated temperatures, but also includes metabolic stress, changes in pH and the disposition of mis-folded proteins (1-6). The association of HSPs with cellular proteins and peptides in an attempt to repair mis-folded proteins has been a major cellular function of constitutive HSPs (7). The repair of mis-folded proteins by HSPs involves multiple roles of HSPs some of which include protease-like activity that aid in the production of correct size peptides so as to serve as ligands for major histocompatibility antigens, HLA, class I and II; ATPase like activity that aids peptide translocations; chaperoning activity that allows the intracellular trafficking of peptides to the various organelles (8-11). The association of the peptides with HSPs prevents the nascent peptides coming of the ribosome from aggregating with other peptides and thus allowing it to fold into the native conformation. The specific property of the HSPs to associate with peptides has been utilized to generate specific tissue derived immunogens.

Specific cancer derived HSPs belonging to HSP70 and gp96 family were found to have specific tumor rejection properties (12,13). Examination of the structure of the HSPs from different tissues did not reveal any sequence differences suggesting similarity of structure of the HSPs derived from different tissues (14). The unique immune stimulatory property that results in tumor rejection was attributed to the peptides associated with the HSPs (15). The HSP-peptide conjugate was the immunogen and neither HSP alone nor the peptides alone were able to confer immune stimulation that resulted in tumor rejection (16-20). Since tumor derived HSPs are associated with normal peptides as well as tumor associated peptides, the optimal treatment with these HSPs requires a careful balance of introduction of immune stimulatory antigens and the elimination of tolerant self-peptides. This kind of approach has prompted an exploration of treatment modalities of metastatic cancer and infectious diseases using HSPs (21).

The use of HSP-peptide complexes as therapeutic agents is dependent on the association of peptides generated in the cytosol. The proteasome that directs the production of peptides consists of a multi-subunit cylindrical complex of molecular mass of 700-750 kDa (22). The proteasome is a protein degradation unit which, unlike proteases, that cleave at only specific sites, has a wide range of simultaneous catalytic protein degradation activities (23-25). As a result of this function, multiple peptides are generated and generation of partially digested polypeptides are minimized. Some of these peptides in the cytosol are transported into the endoplasmic reticulum by specific proteins such as TAP, transporter associated with antigen presentation. Peptides are also associated with HSP70 that is predominantly in the cytosol and these peptide-complexes can also have therapeutic properties.

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Inhibition of proteasome activity results in the accumulation of peptides with mis-folded configuration and is expected to affect cellular HSP levels. Inhibition of proteasome activity is brought about by proteasome inhibitors, several classes of which are available now.

Proteasome inhibitors (PIs) are useful tools to investigate various functions of proteasomes. Most of the PIs are peptide aldehydes, one example of which is MG-132 which is carbobenzoxyl-leucinyl-leucinyl-leucinyl-leucinal which binds reversibly to 20S proteasome (26). Peptide aldehydes also inhibit lysosomal cysteine proteinases. Other proteases such as Calpain which is a Ca^{++}-activated protease is a non-specific inhibitor. Lactacystin, a natural product isolated from streptomyces specifically inhibits 20S proteasomes by modifying threonine residues (27). The inhibition of proteasomes is expected to prolong the half-life of cytosolic proteins that can modulate cell proliferation by interfering with the apoptotic machinery. Such a scenario has been envisioned in instances where degradation of anti-apoptotic proteins is inhibited. This study attempts to examine the status of the HSPs in different cancer cells under condition of proteasome inhibition by MG-132, Lactacystin and a Calpain inhibitor II, LLM. The effect of the PIs was also examined on the growth of the cancer cells and a correlation drawn between growth and cell-type specific activity of the different proteasome inhibitors.

Materials and methods

Cell culture. Cell lines used in this study T2, LG-2 were grown in RPMI-1640, while MCF-7 was grown in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA). The media was supplemented with 10% fetal bovine serum (FBS) (Gemini, Calabasas, CA), 50 IU/ml penicillin (Mediatech, Herndon, VA), 50 µg/ml streptomycin (Mediatech, Herndon, VA) and 2 mM L-glutamine (Mediatech, Herndon, VA). The proteasome inhibitors used were Lactacystin (Calbiochem, La Jolla, CA), MG-132 (Calbiochem, La Jolla, CA) and LLM (Sigma, St. Louis, MO).

XTT assay. XTT assay was performed essentially as described by Scuderio *et al* (28). 2,000-5,000 cells in 100 µl were plated into each well of 96-well plates and incubated for 16-24 h. This is necessary for adherent cells and not for cells that grow as suspension cultures. The media was removed and different proteasome inhibitors were added at desired concentration(s) in a total volume of 200 µl and incubated for 96 h. The media was discarded and fresh growth media added without drug followed by 50 µl of XTT solution [1 mg/ml in serum-free RPMI or DMEM + PMS (5 µl/ml) of XTT before use]. The plate was read after 3-4 h in a microplate reader at 450 and 630 nm.

Western blot analysis. Cells were treated with proteasome inhibitors at concentrations of 10-200 µM for 3 h. At the end of the incubation period the cells were harvested and washed with PBS. The cells were lysed ($1x10^6$ cells/100 µl of lysis buffer) using RIPA buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.5% NP-40, 1 µM Pefabloc], by incubating on ice for 30 min, followed by sonication for 1 min. The lysate was centrifuged at 14,000 rpm for 30 min and supernatant collected. Cell lysates were subjected to 12.5% SDS-PAGE (polyacrylamide gel electrophoresis) (29-31) under reducing conditions (presence of ß-mercaptoethanol). The proteins were transferred to Immobilon-P membranes at 200 mA for 2 h and membranes blocked with 5% milk-PBS-T (phosphate-buffered saline containing 0.5% Tween-20) for 3 h at room temperature on a shaker. Subsequently, the membrane was incubated overnight at 4°C with anti-Hsp70 antibody and anti-grp94 antibody (NeoMarkers, Fremont, CA) on a shaker. Membranes were washed 3 times with 5% milk-PBS-T and incubated with anti-mouse-HRP conjugate (for Hsp70 antibody) and anti-rat-HRP conjugate (for grp94) for 1 h at room temperature on a shaker. After 4 washes with milk-PBS-T and 1 wash with PBS, membranes were developed by ECL (Amersham) and detected on X-ray film.

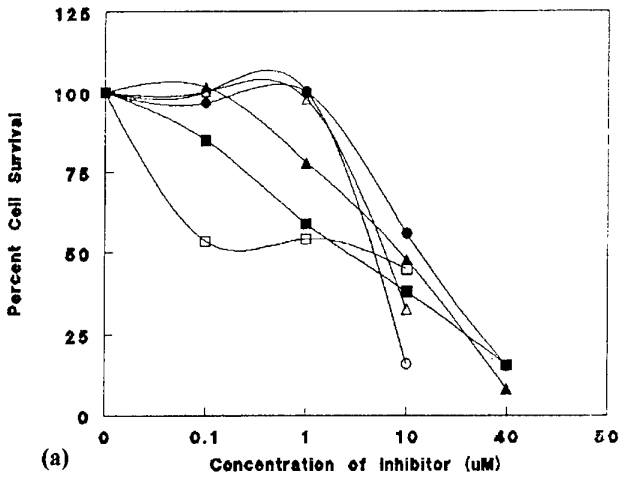
Results

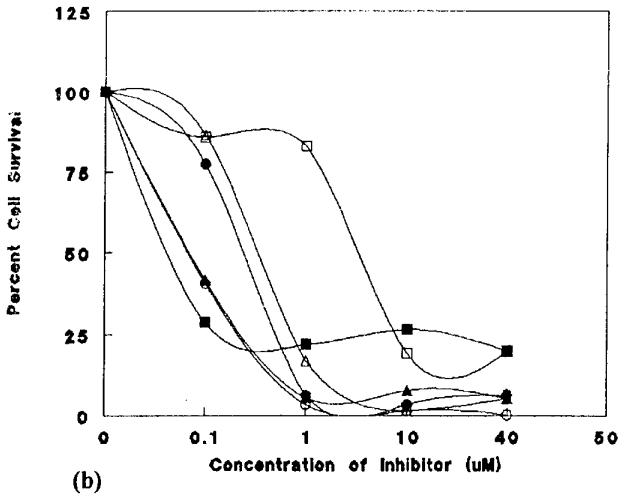
Proteasome inhibitors differentially affect the growth of cancer cells. The effect of three proteasome inhibitors, Lactacystin, MG-132, and LLM, on the growth of several human and rodent cancer cell lines was examined by the XTT assay (Fig. 1). The cancer cell lines included in the experiments were breast cancer cells (MCF-7 and MDA-MB-231), rat prostate cancer cell lines (MLL and DG that correspond to MAT-LyLu and Dunning G) and lymphoblastoid cells LG-2 and leukemic cell line HL-60. The range of the IC_{50} values for Lactacystin was between 4-14 µM with LG-2 being most sensitive and HL-60 being the least sensitive (Fig. 1a). All the epithelial cell lines tested had IC_{50} values intermediate between LG-2 and HL-60 and were not significantly different from each other and ranged from 7 to 9.5 µM (Table I).

The effect of MG-132, however, was in sharp contrast to the cellular effect of Lactacystin. The sensitivities of the cancer cell lines were vastly different from the rat prostate cancer cell lines that were most sensitive (Fig. 1b) and MCF-7 being the least sensitive. Except for MCF-7, all the cell lines exhibited IC_{50} values below 1 µM. The most notable difference was observed between the estrogen receptor positive cell line, MCF-7, and the estrogen receptor negative cell line MDA-MB-231. MDA-MB-231 cells were 10-fold more sensitive than MCF-7 cells. With the exception of MCF-7, all the cancer cell lines were at least 10-fold more sensitive to the growth inhibitory effect of MG-132 than Lactacystin and the IC_{50} values were in 0.1 µM range.

Most of the cancer cell lines tested in our study did not respond to LLM (Fig. 1c). The IC_{50} values were considerably higher, 10- to 20-fold when compared to Lactacystin and 100- to 200-fold when compared to MG-132 (Table I). Amongst the panel of cell lines tested, LG-2 was the most sensitive and MCF-7 the least sensitive. A differential effect was observed between the rat prostate cancer cell line MAT-LyLu and Dunning and between MCF-7 and MDA-MB-231.

Effect of proteasome inhibitors on the steady-state protein levels of HSP70 and gp96 in cancer cells. Western blot analysis using LG-2 cell extracts showed that LLM at 50 and 200 µM induced gp96 very efficiently in 3 h (Fig. 2a). The induction was also observed in the same cell line for HSP70 where there





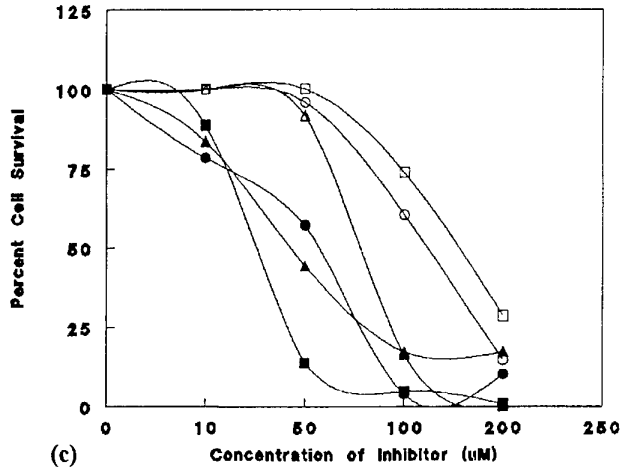


Figure 1. Dose-dependent effect of Lactacystin (a), MG-132 (b) and LLM (c) on the viability of cancer cells as determined by XTT assay. The cancer cells used were MCF-7 (□), MDA-MB-231 (△), MAT-LyLu (○), HL-60 (●), Dunning G (▲) and LG-2 (■). Cells were plated at a density of 2,000 cells/well and after overnight incubation, varying concentrations of proteasome inhibitors were added to each well for 96 h. 50 µl of XTT was added to each well and incubated for 3-4 h and absorbance read using a microplate reader.

Table I. IC_{50} values of cancer cell lines treated with proteasome inhibitors.

Cell line	IC_{50} (µM)		
	Lactacystin	MG-132	LLM
MCF-7	7.1	5.9	157.7
MDA-MB-231	7.9	0.59	78.9
MAT-LyLu	7.2	0.083	123.1
Dunning G	9.5	0.085	43.9
LG-2	4.5	0.062	30.8
HL-60	13.5	0.45	57.7

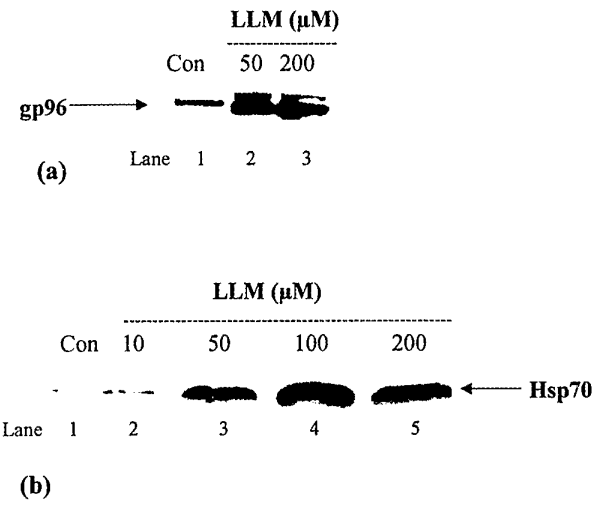


Figure 2. (a), Detection of gp96 in LG-2 cells by Western blot analysis. Cells were either untreated (lane 1) or treated with 50 µM (lane 2) and 200 µM (lane 3) of LLM and lysates (20,000 cells/well) were separated by 12% SDS-PAGE and transferred to PVDF membrane for Western blot analysis. Gp96 was detected using anti-grp94 monoclonal antibody and developed using ECL. (b), Detection of Hsp70 in LG-2 cells by Western blot analysis. Cells were either untreated (lane 1) or treated with 10 µM (lane 2), 50 µM (lane 3), 100 µM (lane 4) and 200 µM (lane 5) of LLM and lysates (20,000 cells/well) were separated by 12% SDS-PAGE and Western blot analysis performed as described above using anti-hsp70 monoclonal antibody.

was a dose-dependent increase in the steady state level of protein (Fig. 2b). The concentrations of LLM used was higher than the IC_{50} values (computed at the end of 96 h exposures) but since these were short-term exposures (3 h) no cytotoxic effect, by visual morphological analysis, was noted. LLM mediated induction at the protein in LG-2 cells was dramatic but similar exposure of LLM to the rat prostate cancer cell line, MAT-LyLu was unable to induce HSP70 (Fig. 3b), and the induction of gp96 was marginal only at

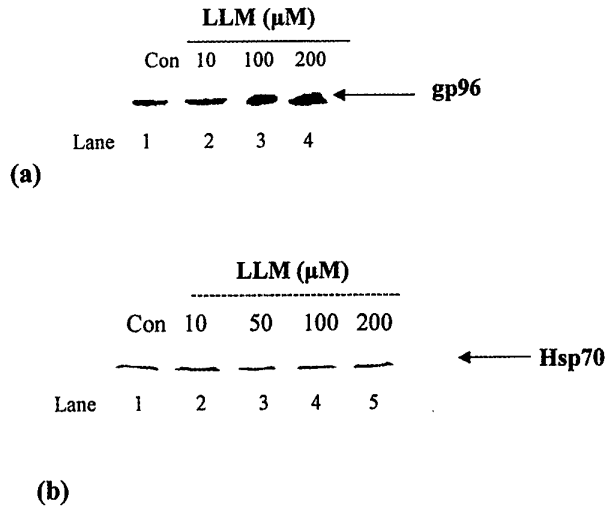


Figure 3. (a), Detection of gp96 in MLL cells by Western blot analysis. Cells were either untreated (lane 1) or treated with 10 μM (lane 2), 100 μM (lane 3) and 200 μM (lane 4) of LLM and lysates (20,000 cells/well) were separated by 12% SDS-PAGE and Western blot analysis performed as described in legend to Fig. 2. (b), Detection of Hsp70 in MLL cells by Western blot analysis. Cells were either untreated control (lane 1) or treated with 10 μM (lane 2), 50 μM (lane 3), 100 μM (lane 4) and 200 μM (lane 5) of LLM and lysates (20,000 cells/well) were separated by 12% SDS-PAGE and Western blot analysis performed.

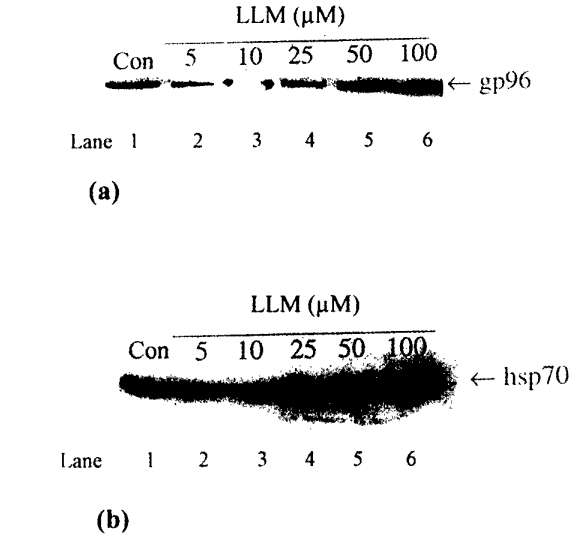


Figure 4. (a), Detection of gp96 in K562 cells by Western blot analysis. Cells were either untreated (lane 1) or treated with 5 μM (lane 2), 10 μM (lane 3), 25 μM (lane 4), 50 μM (lane 5) and 100 μM (lane 6) of LLM and lysates (20,000 cells/well) were separated by 12% SDS-PAGE and Western blot analysis performed as described in legend to Fig. 2. (b), Detection of Hsp70 in K562 cells by Western blot analysis. Cells were either untreated (lane 1) or treated with 5 μM (lane 2), 10 μM (lane 3), 25 μM (lane 4), 50 μM (lane 5) and 100 μM (lane 6) of LLM and lysates (20,000 cells/well) were separated by 12% SDS-PAGE and Western blot analysis performed.

higher doses (100-200 μM). Since LLM mediated induction was higher in LG-2 (lymphoid) than MAT-LyLu (epithelial cells) another leukemic cell line K562 that lacks MHC class I

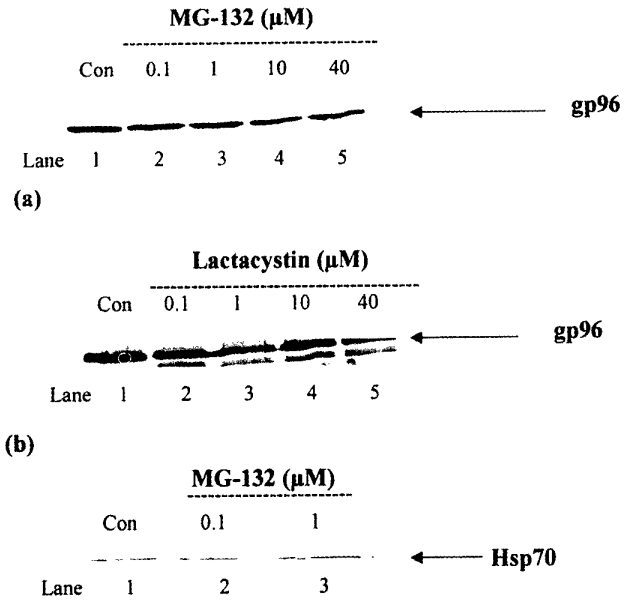


Figure 5. (a), Detection of gp96 by Western blot analysis in MLL cells. Cells were either untreated (lane 1) or treated with 0.1 μM (lane 2), 1 μM (lane 3), 10 μM (lane 4) and 40 μM (lane 5) of MG-132 and lysates (20,000 cells/well) were separated by 12% SDS-PAGE and Western blot analysis performed. (b), Detection of gp96 by Western blot analysis in MLL cells. Cells were either untreated (lane 1) or treated with 0.1 μM (lane 2), 1 μM (lane 3), 10 μM (lane 4) and 40 μM (lane 5) of Lactacystin and lysates (20,000 cells/well) were separated by 12% SDS-PAGE and Western blot analysis performed. (c), Detection of Hsp70 by Western blot analysis in MLL cells. Cells were either untreated (lane 1) or treated with 0.1 μM (lane 2) and 1 μM (lane 3) of MG-132 and lysates (20,000 cells/well) were separated by 12% SDS-PAGE and Western blot analysis performed. Protein concentration used was similar to other experiments (Figs. 2-4), but the blots were deliberately over-exposed to estimate induction levels.

was tested for LLM mediated induction of gp96 and HSP70 (Fig. 4). LLM failed to induce either gp96 or HSP70 in K562 cells up to 100 μM indicating the cell-type specific effect of LLM. MG-132 and Lactacystin also failed to induce gp96 or HSP70 in MAT-LyLu (Fig. 5) although when analyzed for cell proliferative assay these cells were sensitive to both MG-132 and Lactacystin.

Discussion

An analysis of the growth inhibiting effects of proteasome inhibitors in various cell lines suggests a pleomorphic effect of these inhibitors. It is clear from our study that PIs that have specificity of action had the most pronounced effect as revealed by the comparative values of the IC_{50} of the three proteasome inhibitors compared. MG-132, is a specific inhibitor of cysteine proteases. A surprising finding of our results was the differential effect of MCF-7 and MDA-MB-231 suggesting that possibly the presence of estrogen receptor affects either the apoptotic cascade induced by MG-132 or that the estrogen receptor can actively sequester the active compound preventing the build up of optimal threshold concentration to manifest its effect on the proteasome. The observation that non-specific inhibitors such as LLM had to

be used at much higher concentrations is a testimony of the several different redundant pathways that converge to push cell proliferative processes that all need to be targeted at the same time and as such a much higher concentration is required. On the contrary, if specific vital pathways are targeted, inhibitory concentrations can be lowered to achieve optimal reduction in cell growth. The differences in the IC_{50} values in the case of MG-132 is a reflection of the use of specific targets for cell viability and would suggest that sensitive cells cannot activate alternative cell survival pathways.

The role of heat shock response in the presence of proteasome inhibitors has been examined considering the significant role that heat shock proteins play in protein degradation and synthesis. The heat shock response in general is mediated by alterations in the heat shock inducible factors HSF-1, HSF-2 and HSF-3 (32-34). These factors were found to be specifically modulated in avian cells in response to proteasome inhibitors and not protease inhibitors suggesting that ubiquitin-proteasome pathway may be involved in regulating the cellular levels and possibly activity of heat shock proteins (3,33,34). This up-regulation of heat shock response in the presence of proteasome inhibitors such as Lactacystin and MG-132 was attributed to biochemical mechanisms that lead to hyperphosphorylation of the transcriptional factors leading to a facilitative trimerization that helps the binding of the DNA element to the trimerized product and thus induces gene transcription. We were unable to demonstrate induction of gp96 or hsp70 in response to Lactacystin or MG-132 suggesting that under conditions of our experiment and the cell lines examined this hyperphosphorylation event of the heat shock response factors that could eventually lead to induction of heat shock proteins did not occur. Induction of gp96 and HSP70 was, however, demonstrated very efficiently in LG-2 cells and in the epithelial cell line MAT-LyLu the induction was selective only for gp96 and not of HSP70. These results are indicative of complex pathways that may be operative in different cell lines not only for different proteasome inhibitors but also for different heat shock proteins. The inducibility of specific heat shock proteins in different cell lines may also be dictated by relative constitutive levels and a general requirement of higher levels of heat shock proteins. It may be presumed that cell lines that have higher constitutive levels of heat shock proteins would to large extent resist the proteasome inhibitor mediated induction of the HSPs.

In this communication, we examined the inhibition of cell growth with inducibility of HSPs as a first line intermediate biomarker of cell stress response that would set the cascade pathway leading to cell growth inhibition. Specific proteasome inhibitor such as MG-132 was the most potent cell growth inhibitor but was an ineffective HSP inducer and similarly the non-specific inhibitor LLM induced the HSPs very efficiently but was not particularly sensitive to the cancer cell lines. These data suggest a differential cell-type specific effect where the HSP response may be an initial stress response which depending on the cell-type and its stage of differentiation may or may not be translated to cell growth inhibition. The mechanism by which specific proteasome inhibitors affect cell growth mediated by the ubiquitin-proteasome pathway may involve modulation of the HSPs in a cell-type dependent manner. Combination of proteasome inhibitors that can up-regulate HSPs can find use in both chemotherapy and immunotherapies that involve the heat shock proteins.

Acknowledgements

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Preventive and therapeutic effect of tumor derived heat shock protein, gp96, in an experimental prostate cancer model

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Abstract. Tumor-derived purified heat shock protein (HSP), gp96, has tumor protective effect in a number of experimental cancers that include fibrosarcoma, hepatoma, and spindle cell carcinoma. The rationale for using gp96 as a vaccinating agent stems from the discovery that HSPs, including gp96, chaperone antigenic peptides for eventual recognition and elicitation of an immune response. The immune response generated by the HSP-peptide complex is specific to the tumor from which they are derived. The long-term objective of our studies is to develop a vaccine for primary and metastatic prostate cancer using tumor-derived HSPs. In the present study, we report our results on the tumor protective effect of irradiated Dunning G cells, or purified preparations of g96-peptide complexes as a tumor vaccine. Tumor incidence, latency, and tumor growth were the end points of measurement. Tumor bearing Copenhagen rats, made free of disease by surgical resection of the tumors resisted a fresh challenge of live Dunning G tumor cells. Vaccination with irradiated whole cells failed to elicit any resistance to tumor growth. Vaccination with Dunning G derived purified gp96-peptide complexes delayed both incidence and growth of Dunning G induced tumors. Inhibition of tumor growth was observed when gp96 was administered after tumor induction. Our data suggests that tumor derived gp96-peptide complexes can be used as an effective prophylactic and therapeutic agent even in poorly immunogenic cancer such as prostate cancer. Further investigations will determine specificity of action and define the immunological determinants and experimental conditions for its optimal activity.

Introduction

Prostate cancer is the most common malignancy of American men with approximately 41,000 deaths out of an estimated 180,000 new cases diagnosed every year (1). Clinically, the major concern is metastatic disease, which even if detected early is not amenable to standard surgical or chemotherapeutic treatments (2). Since experimental strategies leading to prevention of primary or metastatic disease has obvious clinical implications, the long term objective of our study is to develop prostate cancer specific vaccines for primary prostate cancer and metastatic disease. Our approach assumes the presence of tumor rejection antigens/antigenic epitopes in tumor, an assumption that is based on seminal observations made in cancer immunology over decades of rigorous research spanning different experimental systems that tumor derived molecules can confer protective tumor immunity (3-5). With recent advances in our knowledge that poorly immunogenic cancers can also be exploited to generate an immune response (6,7), immunopreventive and therapeutic approaches using tumor derived preparations, presumably containing tumor rejection antigenic epitopes, have acquired novel clinical significance. Our experimental approach is to develop a cancer vaccine using tumor derived heat shock protein, gp96-peptide complexes, and test its validity in a preclinical prostate cancer experimental animal model.

HSPs include many proteins which are ubiquitously expressed at a basal level but are specifically induced in response to various stress conditions such as heat, anoxia and metabolic stress. In recent years a fundamental role of HSPs in cell homeostasis has emerged. They bind to a myriad of cellular peptide/protein thus acting as molecular chaperones (8-14). In fact, HSPs isolated from tissues are always associated by tightly bound peptides that presumably represent the antigenic repertoire of the tissue of origin. Some of these HSP bound peptides are eventually processed and presented by the mixed histocompatibility complex (MHC) to evoke an immune response. Immunization of mice with any of the three major HSPs (gp96, HSP90, HSP70)-peptide complexes isolated from malignant cells has been found to induce a tumor specific immunity mediated by $CD8^+$T cells (14-20). It is noteworthy that such an immunity is not induced by HSP-peptide complexes isolated from normal cells. This finding, in conjunction with the lack of sequence variation (s) between HSP isolated from malignant and normal cells has been taken as evidence to predict that tumor specific immunity is not induced by HSPs *per se*, but rather from tumor-specific peptides. This prediction has been corroborated by the loss of tumor-specific immunogenicity of HSP stripped of their peptides. These results, which strongly suggest that HSP-

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Key words: gp96, vaccination, rat model, prostate cancer, protective immunity, irradiated cells

peptide complexes isolated from malignant cells represent a useful source of tumor associated peptides, possibly tumor rejection peptides, have provided the background to investigate the prophylactic and therapeutic effect of tumor derived gp96-peptide complexes in a well characterized rat experimental model that utilizes Dunning G cells grown in syngeneic Copenhagen rats.

The R3327/Copenhagen rat model is a valuable experimental system with several advantages despite the obvious disadvantage that the progression of prostate cancer in an animal model does not resemble the human disease. Besides the predictable time and nature of experimental tumors, the major advantage is the availability of a wide variety of cell lines derived from the original tumor with varied tumorigenic and metastatic properties. Of interest to the present study is the Dunning G cell line which is slow growing, androgen responsive and non-metastatic, whereas, in contrast the MAT-LyLu, a subline derived from the G cells, grows three to four times faster than the G subline, is androgen independent and highly metastatic (21).

Results presented in this study indicate that Copenhagen-Dunning G rat model shows tumor-induced protective immunity and is amenable to immunological experimentation. Further, tumor derived heat shock protein, gp96-peptide complexes has a prophylactic and therapeutic effect as determined by tumor incidence, latency and rate of tumor growth.

Materials and methods

Animal experiments. Four- to five-week old Copenhagen rats purchased from Harlan Sprague Dawley (Indianapolis, MN) were placed in groups of three per cage and were provided with food and drinking water *ad libitum* and were placed in 12-h light and 12-h night cycle. The animals were allowed to acclimate one week prior to experimentation. All injections of either irradiated tumor cells, live tumor cells or purified proteins were done subcutaneously. Live tumor cells were injected on the flank of the animal that were shaved prior to injection. Tumor measurements and weight of the animal were monitored weekly. Vaccination with purified gp96 or with irradiated cells was done at day 0 with a booster at day 7 and live tumor cell challenge at day 7. Tumor measurements were done by a vernier calliper and tumor volume expressed as cubic cm was computed using the formula 0.4 x long diameter x (short diameter)^2.

Cell growth. Dunning G cells were grown in culture in RPMI 1640 containing 10% fetal bovine serum (FBS) and supplemented with 2 mM L-glutamine, 50 IU/ml of penicillin and 50 μg/ml of streptomycin and dexamethasone (0.25 μM). Dunning G cells grown at 80% confluence was the source of purification of gp96 that was used as a vaccinating and therapeutic agent. The rat cell lines were a kind gift of the laboratory of John Isaacs (Johns Hopkins, Baltimore, MD).

Purification of gp96. Dunning G cells grown in T-175 flasks were the source of purification of gp96-peptide complexes essentially following the methods described earlier (14). Briefly packed cell volume of 8-10 ml were used in a single purification experiment. Cells were homogenized in a Dounce homogenizer (30-40 strokes) after allowing to swell in five time cell pellet volume of a hypotonic 30 mM sodium bicarbonate buffer. Cell debris and cellular organelles were removed by ultra-centrifugation at 27,000 rpm and the clear supernatant used for gp96 isolation. After two rounds of ammonium sulfate precipitation, 50% saturation followed by 80% saturation, the solubilized precipitate was subjected to column chromatography using Con-A Sepharose, buffer exchange with PD-10 (Pharmacia Biotech, Piscataway, NJ) and ion-exchange with DEAE-Sephacel. The eluted proteins are separated on an SDS-PAGE and the purified protein visualized by silver staining and quantitated by optical density measurements at 280 and 260 nm.

Results

Dunning G cells contain gp96. Fig. 1 shows the step-wise purification of gp96 from Dunning G cells. Although two bands are observed after the DEAE-Sephacel, this material was not purified further as the antigenic composition of both of these proteins have been found to be similar (22). A typical yield of the purified protein was 400 μg protein per 10 ml packed cell pellet. This protein was recognized by the antibody to grp94 (Neomarkers, Fremont, CA) in a Western blot performed by methods described (23), using anti-rat IgG and iodinated protein G (NEN/Dupont, Boston, MA).

Tumor-induced protective immunity is detectable in the R3327/Copenhagen syngeneic model. To determine if Dunning G induced tumors elicit a protective immune response the following experiment was performed. One million live Dunning G cells was injected subcutaneously and tumors allowed to develop until 5 cm^3 (twelve weeks after tumor cell injection). The tumors were surgically resected and the animals allowed to recuperate for two weeks after which they were injected with one million fresh live Dunning G cells on the flank opposite to the previous tumor. As control, three naive rats of approximately the same age were also injected with one million Dunning G cells. As shown in Fig. 2, the naive animals which did not have a prior exposure to tumor cells started developing tumors in about four weeks, whereas, the animals that had prior exposure to Dunning G cells developed tumors by ten weeks, a lag of about six weeks as compared with naive animals. This observation was repeated twice and was consistent, only one experiment is represented in Fig. 2. The delay in tumor induction by fresh cells in animals that had prior exposure to these cells is indicative of a protective tumor response that may be active, albeit only for a limited duration. The degree of immune response, the determinants of the immune response and the specificity of the immune response needs investigation.

Further, we examined if the tumor protective effect can be elicited by irradiated whole cells. Dunning G cells were irradiated with 3000 rads and used as vaccinating agents. Rats were vaccinated with irradiated Dunning G cells at day 0 and day 7 at doses of five and twenty million per rat and then challenged with live cells on day 14. In a separate experiment live cell challenge was done on day 7. Vaccination with irradiated cells offered no tumor protection and the

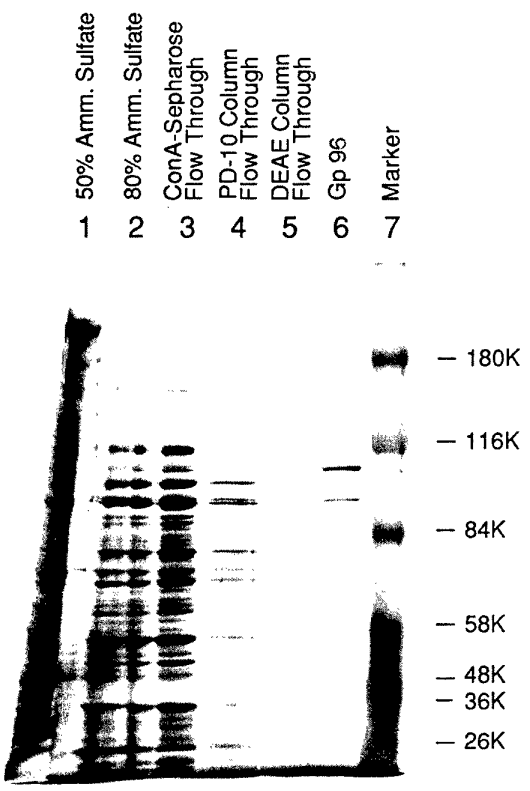

Figure 1. Purification of gp96 from Dunning G cells. SDS-PAGE analysis of cellular proteins followed by silver staining.

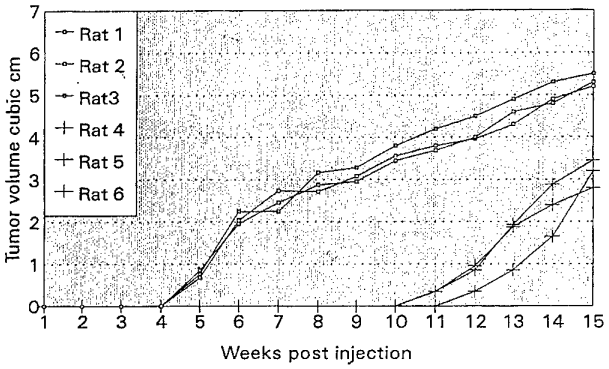

Figure 2. Protective immunity of Dunning G induced tumors in Copenhagen rats. All rats were injected with 1 million Dunning G. Each line represents tumor growth of a single animal; Rats 1, 2 and 3 are naive rats; Rats 4, 5 and 6 had their tumors (3-5 cms) surgically removed and challenged with fresh live cells.

growth rate was comparable in naive and vaccinated animals (data not shown) in any of the experiments. Thus, the agent that mediated the intact-tumor mediated tumor protective effect could not be mimicked by whole cells suggesting the involvement of other intracellular molecules.

Prophylactic effect of tumor-derived gp96. It has been observed in several experimental models that purified preparations of gp96 can protect against subsequent challenges of the tumor cells from which it is derived (19). To test if gp96 isolated from Dunning G cells could protect against Dunning G induced tumors in Copenhagen rats, we vaccinated a group of mice at two different concentrations, 10 and 40 μg per rat, and compared this experimental group with rats that were either sham vaccinated with phosphate buffered saline (PBS) or vaccinated with rat albumin. The vaccination schedule was day 0 and day 7 and live cell challenge with one million Dunning G cells at day 7. The effect of Dunning G derived gp96-peptide complexes on tumor incidence and latency is presented in Table I. By six weeks none of the control animals are tumor free, whereas, 66% of the rats vaccinated with gp96-peptide complexes are tumor free. All rats vaccinated with gp96 show palpable tumors by nine weeks as opposed to six weeks in the non-immunized group, an increase in the latency of three weeks. The tumor inhibitory effect is also reflected on the rate of tumor growth (Fig. 3) and the tumor volume (Table II). Reduction in tumor volume of 50% is observed in the gp96 vaccinated group as compared to the sham vaccinated group. Statistically significant reduction of tumor growth was observed in the rat albumin vaccinated group when compared with control. Rat albumin has peptide binding property and

Table I. Effect of immunization of gp96-peptide complex isolated from Dunning G cells on tumor incidence in syngeneic Copenhagen rats.

	No. of weeks post challenge of $1x10^6$ live Dunning G cells					
	5	6	7	8	9	10
Control (non immunized)	4/5	5/5	5/5	5/5	5/5	5/5
gp96 (10 μg/rat)	2/6	2/6	2/6	3/6	3/6	6/6
gp96 (40 μg/rat)	0/6	2/6	3/6	3/6	6/6	6/6
Rat albumin (40 μg/rat)	5/5	5/5	5/5	5/5	5/5	5/5

Table II. Average tumor size mean ± SD cm^3.

Control	5.08±0.30	
gp96 (10 μg/rat)	2.27±0.68	p<0.01
gp96 (40 μg/rat)	2.20±0.20	p<0.01
Rat albumin (40 μg/rat)	3.80±0.14	p<0.01

All statistical comparisons were done with control values using a Student's t-test and p<0.01 was considered statistically significant.

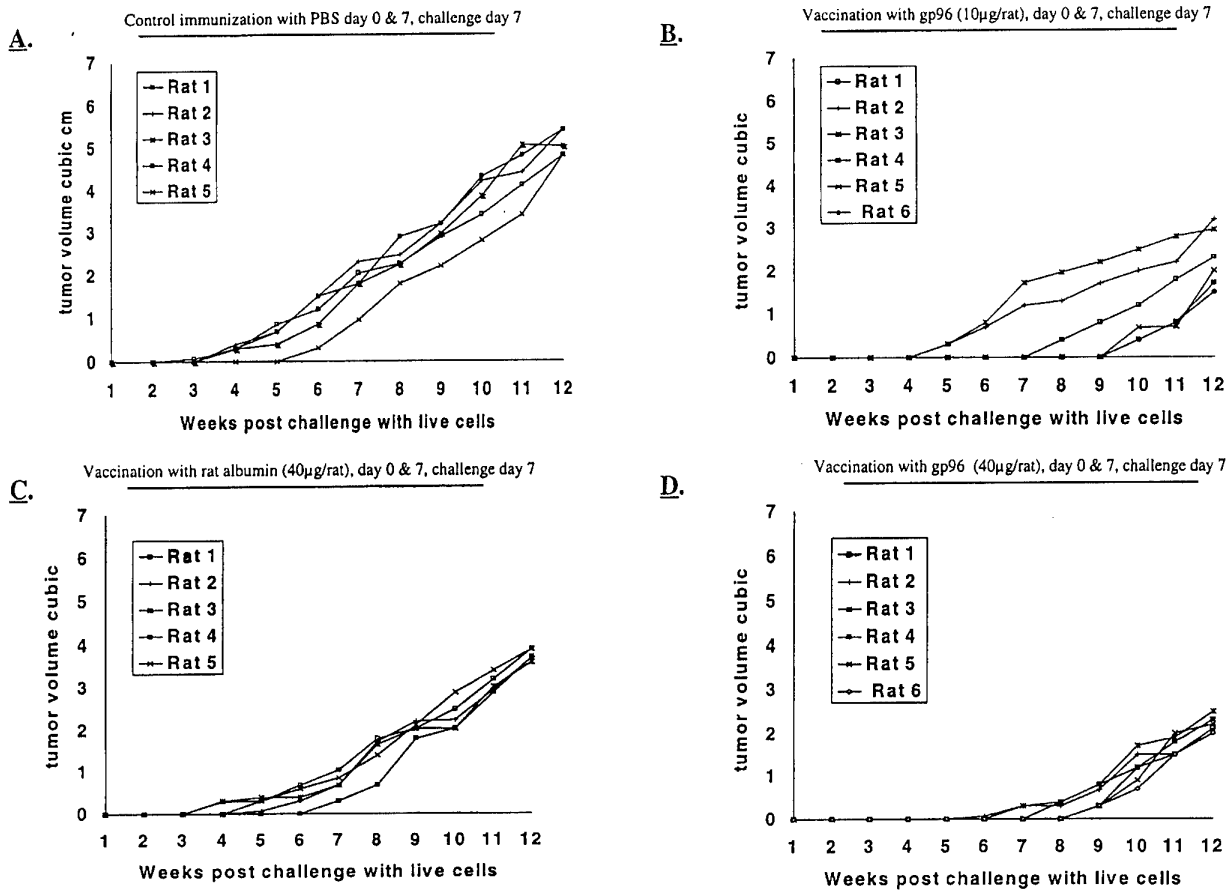

Figure 3. Prophylactic effect of purified gp96 at doses of 10 and 40 µg per rat (panels B and D) as compared with sham vaccination with PBS (panel A) and rat albumin (panel C). Each animal is represented on a single curve.

may not be the ideal control. Animals vaccinated with gp96 from liver tissues showed no protective effect (data not shown). Animals vaccinated with gp96 showed statistically significant reduction in tumor growth when compared with the rat albumin vaccinated group ($p<0.01$). These results provide strong evidence that gp96-peptide complexes can delay tumor latency and alter tumor growth, presumably by alterations of specific immunological response, however, the conditions that can elicit complete tumor regression and render animals vaccinated with gp96 completely tumor free have yet to be determined.

Therapeutic effect of gp96. Administration of gp96 therapeutically has immediate clinical implications. We tested the therapeutic effect of Dunning G-derived gp96 on Dunning G induced tumors in Copenhagen rats (Fig. 4). The therapy was started two and a half weeks after live tumor cell injection. There was no evidence for palpable tumor at initiation of therapy but tumors were predicted to develop in the next two to three weeks. Ten micrograms of Dunning G derived gp96, rat albumin or sham injection of PBS (control animals) was administered to these animals, three times a week for six weeks. Of the four animals that were treated with gp96, three animals showed a reduction or stabilization in tumor growth (Fig. 4). Withdrawal of therapy resulted in the rate of tumor growth comparable to the sham or albumin injected animals. The therapy experiment was also repeated with similar results and only one of the experiments are represented in Fig. 4.

Discussion

Although an experimental animal model that represents the progression of the human prostate cancer does not exist, the R3327 animal model has served a useful purpose in testing the efficacy of various immunological and chemical agents (21,24). The major advantage of this model is the availability of well defined cell lines with predictable tumorigenic and metastatic phenotype, however, the immunological characteristics of the non-metastatic G subline or the metastatic MAT-LyLu has not been examined. We present evidence that tumor induced protective response can be generated in the Dunning G rat model suggesting that preventive and therapeutic strategies using immunological approaches is an option for prostate cancer. A logical avenue for the search of immunological principles that can modulate tumor protective response is the tumor itself, precedence for which exists in defining tumor derived cancer rejection antigens (3-5).

The heat shock protein, gp96, is one such molecule which was discovered as a tumor rejection antigen. After almost

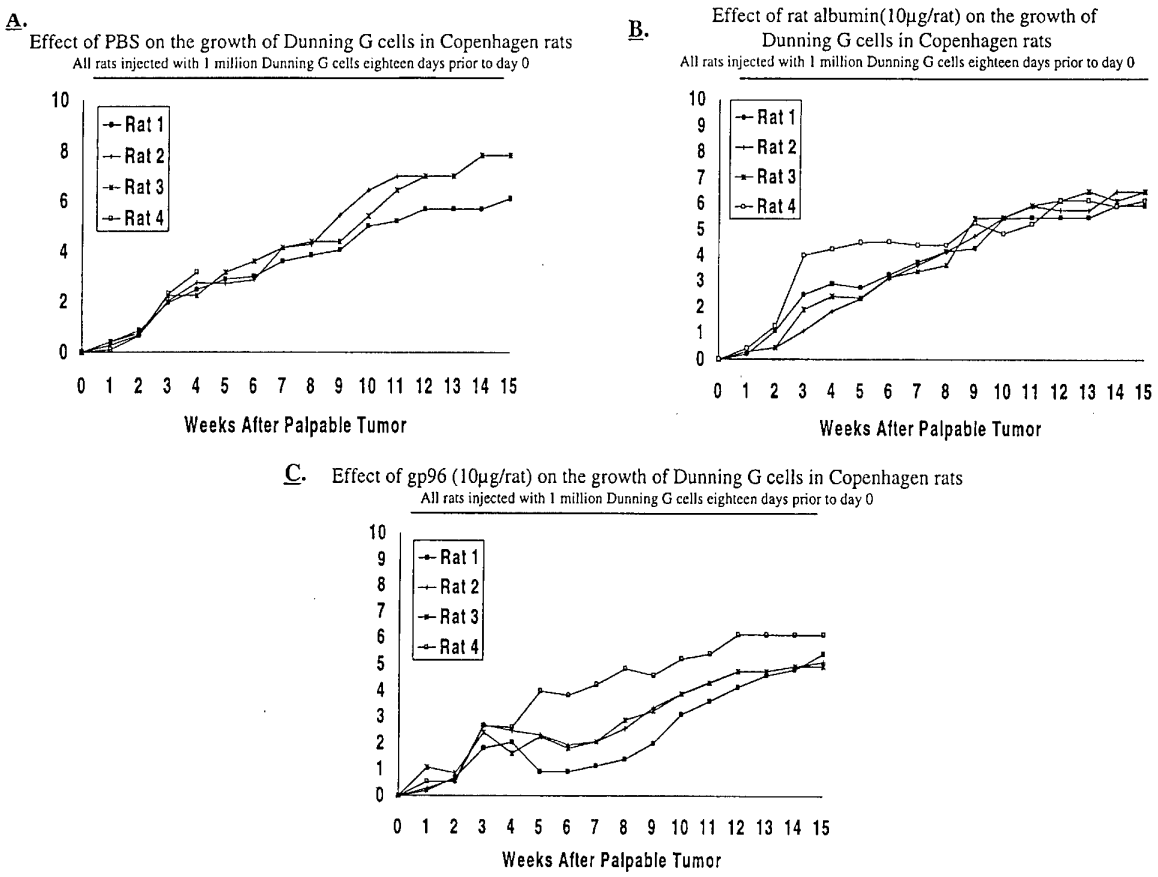

Figure 4. Therapeutic effect of purified gp96 at a dose of 10 μg per rat (panel C) as compared with sham treatment (panel A) and rat albumin at 10 μg per rat (panel B). Each line represents tumor growth of a single animal. Animals were injected with PBS, rat albumin or gp96 subcutaneously three times a week for six weeks.

ten years of intense study, it is now accepted that the immunogenic principle is not the gp96 protein but the complex consisting of gp96 and the myriad of peptides associated with it. It is the peptides that impart specificity of its immunological properties and the enormous diversity of cellular peptides creates the uniqueness of the gp96-peptide complexes that presumably represent a repertoire of cellular antigenic epitopes. The antigenic epitopes represent the entire family of unique, shared and non-specific normal cellular antigens. The presence of unique antigens probably causes tumor regression or suppression and would be most effective clinically, while a gp96 preparation that is only marginally associated with unique antigens and mostly shared antigens would have a reduced level of tumor rejection property. In the case of Dunning G induced tumors, vaccination with gp96 produces a 50% reduction in tumor growth. This may be due to the preponderance of shared antigens over unique antigens in Dunning G derived gp96 preparations. Since similar results were obtained on two separate replications of this experiment it may be assumed as a feature of the tumor system rather than purification artifacts. Alternatively, lack of persistent immune response or a large tumor bolus may be responsible for only a partial regression of these tumors. Careful study with gp96 doses with varying tumor cell challenges will clarify the issue and measurement of the induction of the immune response. Optimization of the vaccination schedule both in terms of number of vaccination, site of injection and the interval between each injections will further determine the extent of gp96-induced tumor regression. Results presented here lay the foundation that preventive immunological approaches can be undertaken with peptide chaperone such as gp96. Identification of the peptides, the immunological principle would also facilitate a standardized vaccination schedule and is being currently being actively examined using a synthetic combinatorial antibody phage display library.

The use of purified gp96-peptide complexes serve as an important repository for the identification of immunologically relevant peptides. Since our data suggests a therapeutic effect as well, the possibility that active specific immunotherapy can be developed with either gp96-peptide complexes or peptides derived from them and natural adjuvants. The selection of specific tumor associated peptides for active immunotherapy for cancer has relied on its tumor cell restricted expression and their ability to function as targets of cytotoxic T lymphocytes (CTL) *in vitro* (6,25). Neither of these criteria identifies tumor rejection antigens/peptides, which may explain the limited success of the use of these immunogens in the clinic. It is our working hypothesis that tumor derived HSP, gp96-peptide complexes that mediate specific protective

tumor immunity are an important source for the identification of tumor rejection antigenic peptides and when combined with gp96 will be a unique preventive and therapeutic agent in prostate cancer.

Acknowledgments

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Anti-tumor effects of PC-SPES, an herbal formulation in prostate cancer

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Abstract. Prostate cancer is the most common cancer amongst males in developed countries. Surgical removal of the prostate effectively cures the primary disease but the metastatic disease is refractory to most forms of chemotherapy. There is a clinical need to develop novel treatment strategies that exploit the mode of action of both conventional and alternative drugs/medicinal plants. We have been investigating the anti-proliferative and anti-tumor effects of an herbal preparation termed PC-SPES (patent pending, US serial number 08/697, 920) which is a refined powder of eight different medicinal plants. PC-SPES administered as a food supplement caused a dramatic decrease in prostate specific antigen levels in some prostate cancer patients with advanced disease. These preliminary clinical findings laid the foundation for a program to examine the *in vitro* and *in vivo* effects of PC-SPES, and identify the active component in this mixture so that a standardized treatment regimen can be formulated. In this communication, we report the anti-tumor effects of PC-SPES incorporated in the diet utilizing a well studied Dunning R3327 rat prostate cancer model. Dietary PC-SPES at levels of 0.05% and 0.025% did not exhibit any toxicity and no significant difference in food intake was noted at the end of six weeks. Dose dependent inhibitory effect of dietary PC-SPES was observed on both tumor incidence (P=0.01) and rate of tumor growth when tumors were induced in syngeneic Copenhagen rats by intradermal injections of MAT-LyLu cells that are known to metastasize in the lung and lymph nodes. The number of pulmonary metastases in animals on PC-SPES that showed no primary tumor growth had no metastatic lesions in the lung, however, in animals that did not respond to PC-SPES, the number of pulmonary metastases was not significantly different from the non-treated controls. The significant anti-tumor effects of PC-SPES on MAT-LyLu induced tumorigenesis and metastasis in Copenhagen rats, in general refractory to most conventional therapy, suggests a therapeutic benefit of this herbal food supplement and may be a useful adjuvant to conventional therapeutic modalities.

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Key words: rat model, anti-tumor effects, PC-SPES, toxicity, pulmonary metastases

Introduction

Prostate cancer accounts for over 180,000 new cases with approximately 40,000 deaths among American men every year. Removal of the prostate is curative for the primary disease with a substantial decline in the quality of life but metastatic prostate cancer is refractory to most prevalent forms of radiation and chemotherapy (1,2). Furthermore, conventional treatments that employ physical or chemical hormone deprivation leads to a more aggressive, rapidly proliferating subset of prostate cancer cells that are hormone independent and presumably acquire a distinct set of growth factors that mediate androgen-independent proliferation (3,4). Specific therapy that targets the functional activity of the epidermal growth factor receptor (EGFR) and the oncogene product HER-2/*neu* are in clinical evaluation but a combination treatment schedule that can target all the subsets of prostate cancer cells is still elusive. Since regulation of cell cycle, induction of apoptosis and steroid hormone induced signal transduction pathways and invasion of the extracellular matrix by enhanced tumor vasculature are all involved in the growth, maintenance and spread of prostate cancer, it is imperative that successful treatment strategy needs to target all or several of the growth regulating pathways simultaneously.

A multi-targeted approach to prostate cancer can be promulgated in several ways. Combination chemotherapy with drugs that target the cell cycle and angiogenesis, or radiation and chemotherapy, or anti-hormone therapy with growth factor/signal transduction inhibitors have shown promise but the success of each novel approach depends on its toxicity, morbidity and nature of the prostate cancer (5-8). Since it is not always possible to examine the phenotypic characteristics of prostate cancer cells by non-invasive procedures, the treatment options are postponed until the appearance of overt clinical or pathological symptoms. At this point potential treatment benefits are significantly reduced. It is therefore important to focus research attention on the prevention of progression of benign disease utilizing a non-toxic, multi-targeted approach.

We have been investigating the anti-tumor and anti-proliferative effect of PC-SPES, a combination of eight different medicinal plants each having a distinct clinical effect (9-11). A dramatic drop in the prostate specific antigen (PSA) levels was observed in some advanced prostate cancer patients. PC-SPES is a refined powder containing *Ganoderma lucidium Karst*, *Dendranthema morifolium Tzvel.*, *Glycyrrhiza glabra L*, *Isatis indigotica*, *Panax pseudo-ginseng Wall*, *Rabdosia rubescens*, *Scutellaria baicalensis Georgi*, and *Serenoa repens* (11,12). Several of these components exert significant biological effects for e.g. *Ganoderma lucidium Karst* exerts a strong immunomodulatory effects and the immunomodulatory protein LZ-8 has been identified (12); Baicalein a flavonoid derived from *Scutellaria baicalensis* has anti-proliferative and lipooxygnesase-inhibitory activity (13); *Serenoa repens* is a potent phytoestrogen and its lipid extracts is an inhibitor of both type 1 and type 2 5α-reductase, the enzyme that converts testosterone to dihydrotestosterone, the active androgen in the prostate (14); Rg 1, a saponin derived from *Panax ginseng* has mitotic activity towards T cells similar to Concanavalin A (15,16) and ginseoside-Rb2 inhibits angiogenesis in a transplantable tumor model; extracts of *Glycyrrhiza glabra* has been shown to have anti-mutagenic activity (17,18). It is the combination of several such potent medicinal plants that presumably exert a synergistic anti-proliferative effects by acting on multiple targets simultaneously.

We examined the effect of PC-SPES in the R3327 syngeneic Copenhagen rat model using MAT-LyLu cells. The advantage of this model is that several cell lines have been developed from a spontaneous tumor that arose in the Copenhagen rats with varied properties. The parental G sub-cell line is androgen responsive and grows slowly and is not known to metastasize. A variant of this cell line termed as MAT-LyLu is rapidly proliferating, is androgen unresponsive and metastasizes to the lung and lymph nodes (19). Mat-LyLu cells have a doubling time of 8 to 10 h and have a 100% tumor take in syngeneic Copenhagen rats and are in general refractory to most conventional forms of therapy.

In the present study we examined the effect of dietary PC-SPES at concentrations of 0.025% and 0.05% on MAT-LyLu cell line induced tumorigenesis in Copenhagen rats. End-points of investigation included tumor incidence, rate of tumor growth, pulmonary metastasis, tumor histology and toxicity and palatability of PC-SPES containing diet. The concentration of PC-SPES have been computed based on the doses used in the clinic and doses that can be achieved by dietary supplementation. Our results suggest that PC-SPES affects tumor incidence, rate of tumor growth and pulmonary metastasis. Animals may be classified as responders and non-responders with complete resistance to tumors in the responders. Animals that showed no tumor were free of metastasis suggesting that both processes of lodging at the primary site and at metastatic site are affected by PC-SPES treatment.

Materials and methods

Growth of MAT-LyLu cells in vitro and in vivo. MAT-LyLu cells (a gift from John Isaacs Laboratory, Johns Hopkins, Baltimore, MD) were grown in RPMI-1640 containing 10% fetal bovine serum (FBS) supplemented with penicillin (50 IU/ml), streptomycin (50 μg/ml), 2 mM L-glutamine and 2.5 mM dexamethasone. Cells were fed twice a week and were trypsinized with 0.05% trypsin-EDTA at 80 to 90% cell confluency. Since the cells are rapidly growing, care is taken that the cells do not reach 100% confluency. Cells injected for growth in Copenhagen rats were generally taken from flasks that were between and 50 and 75% confluent. MAT-LyLu cells were washed twice with phosphate buffered saline (PBS) and then trypsinized and suspended in PBS at a concentration of 100,000 cells per ml. Each animal was injected with 0.1 ml of cell suspension with an effective dose of 10,000 live MAT-LyLu cells per rat. Cell viability was always determined by the trypan blue exclusion test and samples that exhibited less than 98% viability were discarded. Cells were injected into the right flank of the animal which has shaved prior to the injection. All injections were intradermal (i.d.), using an insulin syringe. Cells were kept at 4°C at all times prior to injections. This method of injections with MAT-LyLu, in our experience, yields 100% tumor incidence. All experimental groups and the control animals were injected at the same time with the same batch of cells.

Animal experiments. Four- to five-week old Copenhagen rats were purchased from Harlan Sprague Dawley, Indianapolis, IN, and allowed to acclimate for one week, feeding on Purina 5001 rat chow. At the end of one week the rats were randomized into three groups of seven rats. Groups were fed either Purina 50001 rat chow (control) or Purina 5001 rat chow containing 0.05% or 0.025% PC-SPES. The rats were allowed to continue on the experimental diet for two weeks and their food intake and body weight monitored. Food intake was determined by giving pre-measured amount of food and monitoring consumption of food by weighing the remaining food every alternate day. Body weights of the animals were measured twice a week. Average food consumption per animal per day from each group was compared. This computation also took into account the food wastage of each animal in each of the groups that was in the range of 4.2-4.4 g per rat per day. Live MAT-LyLu (10,000 cells/rat) were injected in all animals on day 14 after randomization into groups. All animals were individually housed in hanging cages and had *ad libitum* access to food and drinking water and were kept on twelve hour diurnal cycle. All injections and tumor measurements were performed under light anesthesia (metofane inhalation). Experimental end point measurements included, dietary intake, body weight gain, tumor incidence, rate of tumor growth, number of animals with pulmonary metastases and number of visible metastatic nodules per lung of the tumor bearing animal and histopathological examination of the tumors. Tumor diameter was measured using vernier caliper.

Dietary formulation. PC-SPES was obtained from BotanicLab. (Brea, CA) and was incorporated into rodent chow 5001 by Purina Labs. (10). Three different concentrations 2.5 g (0.25%), 0.5 g (0.05%), 0.25 g (0.025%) of PC-SPES per kg were formulated. The concentration of 0.05% corresponded to the supplementation to prostate cancer patients. In a separate experiment (not shown here) diets containing 0.25% PC-SPES

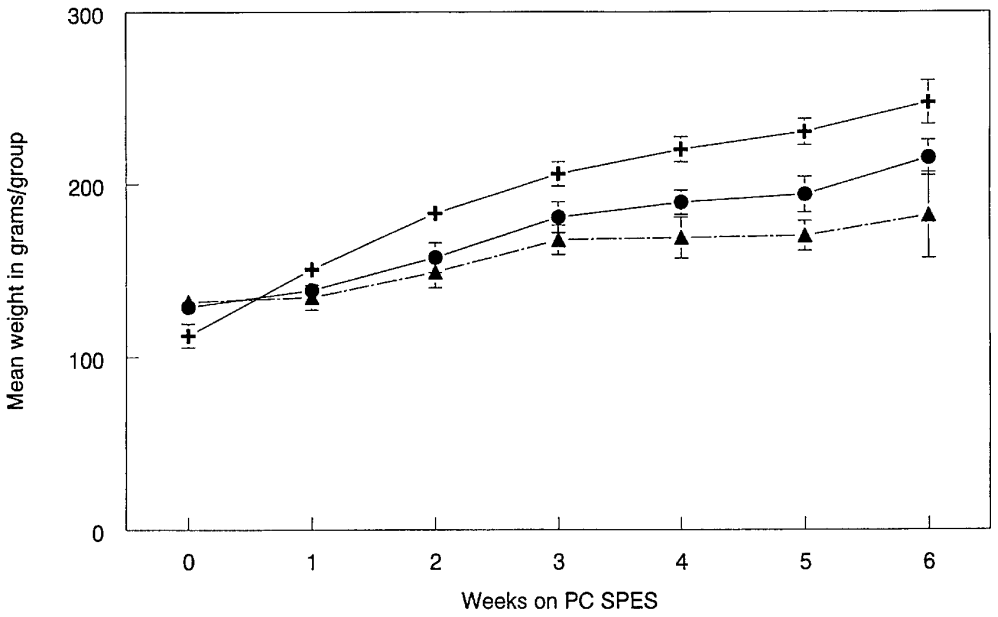

Figure 1. Variation in mean weight in grams/group in Copenhagen rats fed PC-SPES. +, control; ●, 0.025% SPES; ▲, 0.05% SPES.

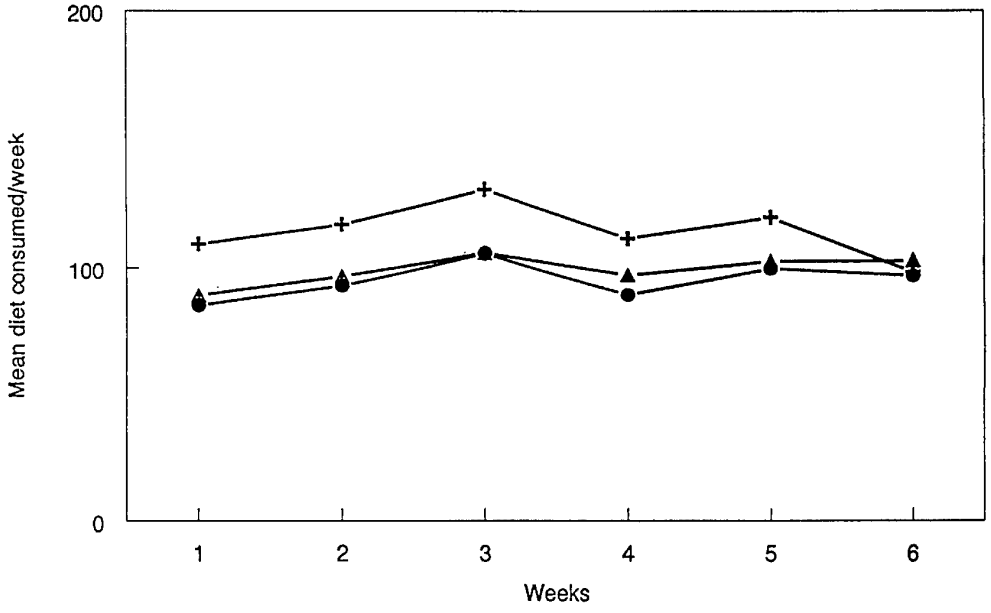

Figure 2. Mean diet consumed/week/group by Copenhagen rats on PC-SPES. +, control; ▲, 0.025% PC-SPES; ●, 0.05% PC-SPES.

was found to be non-palatable in the case of Copenhagen rats as 50% of the animals were found to be malnourished due to refusal of food. Taste was not an issue in the case of prostate cancer patients as they received PC-SPES in capsules. These experiments formed the rationale for the use of 0.05% and 0.025% of PC-SPES in our experimental group.

Statistical analysis. Data was analyzed using a two-tailed Student's t-test and mean values differing from each other at P=0.05 was considered as significant.

Results

Effect of PC-SPES on the body weight of Copenhagen rats. Four to five week old Copenhagen rats were maintained for two weeks on Purina 5001 rodent chow containing PC-SPES at 0.05% and 0.025%, for acclimatization. Change of mean body weight per group (control, 0.05% and 0.025%) is presented in Fig. 1. The mean food intake per group in grams in the different groups is also given in Fig. 2. It is clear that animals on PC-SPES had a decrease in the amount of food

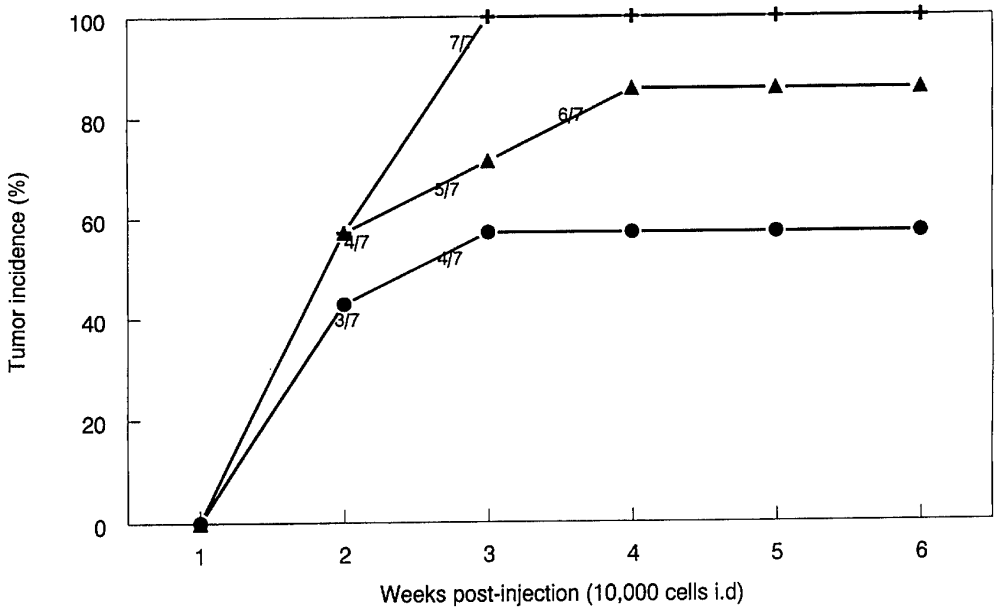

Figure 3. Tumor incidence of Mat-LyLu in Copenhagen rats on PC-SPES. +, control; ▲, 0.025% PC-SPES; ●, 0.05% PC-SPES.

ingested both at 0.025% and 0.05% groups as compared with the control (P=0.002) in the first four weeks of the start of the dietary treatment. The lowered food intake is also reflected in the body weight of the animals that is lower in the PC-SPES treated group in a dose dependent manner. The animals on PC-SPES were found to exhibit normal activity with no hyperactive behavior when compared with the animals on normal diet that contained no PC-SPES. Animals on PC-SPES at 0.05% ingested doses of PC-SPES similar to prostate cancer patients on three 333 mg capsules three times a day. No reports of weight loss was reported in these patients (personal communication, manuscript in preparation), on the contrary some patients had either a significant weight gain or had maintained a steady body weight. This reflects the effect of PC-SPES on the general well being of the prostate cancer patients on this supplement. It should be mentioned that on a dose as high as 0.25% PC-SPES (unpublished observations and data not shown), the animals were malnourished as they refused the supplemented food. It is presumed that such a high concentration of PC-SPES interfered with the palatability of the chow to Copenhagen rats. The fact that 0.05% dose was well tolerated by these animals with no significant overt toxicities as determined by hair loss, loss of appetite, lack of locomotion suggests that supplementation of PC-SPES at this dose assuming similar metabolism in rats and humans should not produce any toxicities in humans. In fact supplementation of PC-SPES at this dose in human patients is well tolerated by prostate cancer patients.

Effect of PC-SPES on tumor incidence in Copenhagen rats. Results presented in Fig. 3 shows the dose dependent effect of PC-SPES on tumor incidence in Copenhagen rats, when tumor was induced by i.d. injections of 10,000 live MAT-LyLu cells. PC-SPES incorporated in the diet at 0.025% resulted in a 20% decrease in palpable tumors in animals, whereas, when the dose of PC-SPES in the diet is increased to 0.05% tumor incidence was decreased by more than 40% (P=0.05). Similar observations were obtained earlier in another experiment with five animals per group. Thus the observations presented here are reproducible and seem to reflect the biological effect of ingested PC-SPES. Dose dependent effect of PC-SPES on tumor incidence suggests that there appears to be responders and non-responders even in inbred strain of experimental animals. Copenhagen rats on PC-SPES diet with no evidence of primary tumor showed no pulmonary metastasis. All the animals in the control showed primary tumor as well as pulmonary metastases. The effect on tumor induction as observed in reduction of tumor incidence at both 0.05% and 0.025% PC-SPES together with the observation that animals that did not have primary tumor lacked pulmonary metastases has significant implication in the preventive use of this herbal supplement provided long term use of PC-SPES does not have *in vivo* toxicity or any side effects. It is also clear from our experiments that doses below 0.025% PC-SPES may not have any significant impact on tumor incidence and hence pulmonary metastasis suggesting an optimal threshold cellular concentration prior to manifestation of its biological effect.

Effect of PC-SPES on the rate of tumor growth and pulmonary metastases in Copenhagen rats. Dose dependent decrease in the tumor burden was noted in animals that were administered PC-SPES. The reduction was noted three weeks after tumor injection with increasing difference in rate of tumor growth inhibition. The mean values were lower than the control as much as 50% [compare mean values of 0.05% treated animals 1.35 to 2.98 in control (Fig. 4)]. A wide range in the tumor sizes was observed in the experimentally treated animals and as such the values did not attain statistical significance. Nevertheless the wide difference in the mean values together with the observation that some animals were devoid of tumors suggests a differential action of this herb and in

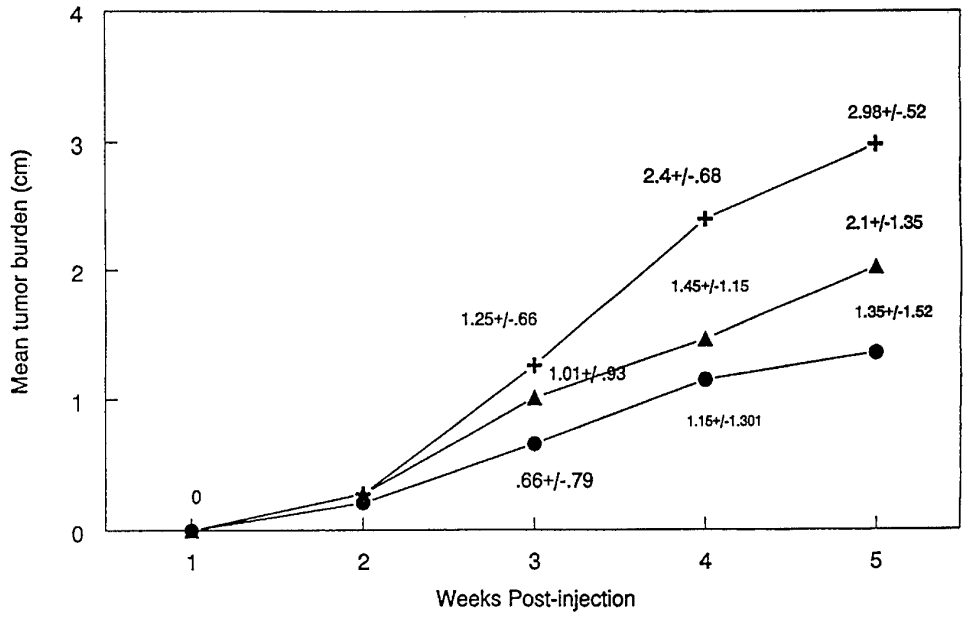

Figure 4. Mean tumor burden/group. Weeks post-injection. +, control; ▲, 0.025% PC-SPES; ●, 0.05% PC-SPES.

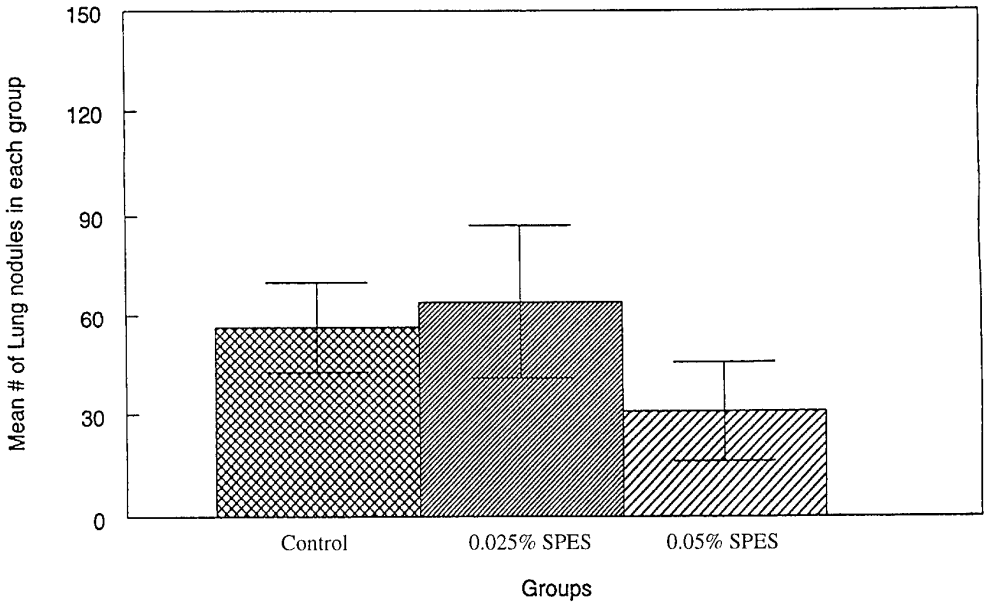

Figure 5. Effect of PC-SPES on lung metastasis.

animals which are responders or partial responders an anti-tumor effect is observed. Animals that received 0.05% PC-SPES showed a plateau in the growth rate, however animals that were scored positive for the primary tumor also showed lung metastasis. Statistically significant decrease (P=0.05) in pulmonary metastasis was observed in the PC-SPES treated (0.05%) when compared with the untreated controls (Fig. 5). It should be noted that 20% of the animals in 0.025% and 40% of the animals in the 0.05% PC-SPES treated animals did not exhibit any lung metastasis. Similar to the effect of PC-SPES on tumor growth the number of macroscopically visible lung metastases showed considerable variation, however, the mean values in the PC-SPES treated control were found to be statistically different from the control group of animals.

Discussion

Effect of PC-SPES on the growth of Copenhagen rats. PC-SPES at doses of 500 and 250 parts per million was well

tolerated by Copenhagen rats with no overt toxicity symptoms. A difference in the mean amount of food consumed can be attributed to peripheral rodent identifiable factors such as taste, odor or texture. These results are contrary to results obtained with PC-SPES supplementation in a capsular form in prostate cancer patients where either a weight gain or stabilization but no weight loss was observed. Since palatability and taste was not a factor in the human population, it is presumed that taste was the major determinant in lowered food intake.

Anti-tumor effects of PC-SPES. Our studies using Copenhagen rats and the transplantable, rapidly proliferating, highly metastatic cell line, MAT-LyLu indicates that dietary supplementation with the Chinese herbal preparation of PC-SPES can modulate tumor incidence, rate of tumor growth and pulmonary metastases. These results are of special significance in the MAT-LyLu model as growth of these cells are in general refractory to most forms of treatment. Decrease in tumor incidence in a dose dependent manner suggests that some animals respond to the anti-tumor effect of PC-SPES better than others and that even in genetically identical inbred strains of rats we may be able to identify PC-SPES responders and non-responders. This is reminiscent of the human experience with PC-SPES where some patients showed dramatic response or were partial responders, while others were completely refractory (Mittelman A, *et al*, manuscript in preparation). Although the precise mechanism for such an action is elusive, one can speculate that since PC-SPES is a mixture of several different medicinal plants, each with its target of action, induction of a synergistic response may be host dependent (11). While published data on the biological effects of PC-SPES suggests that specific ethanol extracts of PC-SPES induces apoptosis and cell cycle deregulation predominantly a prolongation of the G1 phase of the cell cycle, immunological modulation by induction of tumor specific cytolytic T cells by specific component(s) of PC-SPES in its *in vivo* effect cannot be ruled out (11).

The anti-tumor effect of a non-toxic dietary supplement of combination of medicinal plants suggests the relevance of the use of combination therapy that is capable of utilizing multiple targets. These multiple targets consist of biochemical intermediaries that mediate the cell cycle transition by ethanol extracts of PC-SPES (11). Components of PC-SPES can target specific biochemical targets e.g. *Glycerrhiza* can reverse mutations (20), *Serenoa repens*, a phytoestrogen lowers estrogen levels and affects androgen receptor binding with its ligand (14,21,22), *Scuttelaria baicalensis* that contains baicalin inhibits lipooxygenase and DNA topoisomerase activity (23,24), extracts of *Ganoderma lucidum* have potent immunomodulatory effects (12), *Panax ginseng,* probably the most studied herb, has anti-carcinogenic effects on spontaneous and carcinogen induced tumor model systems as well as can affect tumor metastases (11,25). Thus, while most of the active components individually have anti-tumor effects, several different herbs in a mixture in the Chinese and Japanese traditional medicine has been found to be more efficacious. The generation and success of popular mixtures such as 'sho-saiko-to' and 'juzentaihoto' for hepatocellular diseases and enhancement of drug induced anti-neoplastic effects in China and Japan relates to the effectiveness of combination therapy. Each individual component, though potent in their individual effects cannot compare with the effect of the combination preparation (25,26). These anecdotal observations support the hypothesis that preparation that contain mixtures of individually potent drugs have enhanced efficacies that may be related to either synergism of the individual components or the simultaneous hit on multiple cellular and biochemical targets that not only eliminates existing malignant cells but can also prevent the clonal expansion of neoplasms and their further differentiation and thus prevention of secondary metastasis. A recent report of the use of PC-SPES examined the estrogenic effect of PC-SPES in an animal model and in eight patients (27). While the substantial decrease in prostate specific antigen correlated with our observations, the doses used were 'too high' and thus the validity of the estrogenic effect was questioned.

Standardized treatment using PC-SPES. The long-term goal of our studies is to identify the active component(s) of herbal preparations that have shown anti-tumor effects. PC-SPES is one such preparation that has similarities with other Chinese and Japanese preparations from medicinal plants but is unique in other respects. Evaluation of the water soluble and ethanol extracts of PC-SPES in *in vitro* and *in vivo* studies, and separation of the active components using high performance liquid chromatography (HPLC) and examination of the cellular and molecular effects will help development of a standardized drug-like treatment procedures. These studies will help alternative medicinal plant based therapy to enter into the field of molecular medicine. Furthermore, identification of the cellular and molecular targets of the active component of PC-SPES will provide an intermediate biomarker that can presumably evaluate the efficacy of the treatment and or compliance to a treatment schedule.

Acknowledgements

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